VU VRIJE UNIVERSITEIT AMSTERDAM

VU Research Portal

Respiratory behaviour of the pulmonate freshwater snail Lymnaea stagnalis and its neural control

van der Wilt, G.J.

1992

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
van der Wilt, G. J. (1992). *Respiratory behaviour of the pulmonate freshwater snail Lymnaea stagnalis and its neural control*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

E-mail address:
vuresearchportal.ub@vu.nl

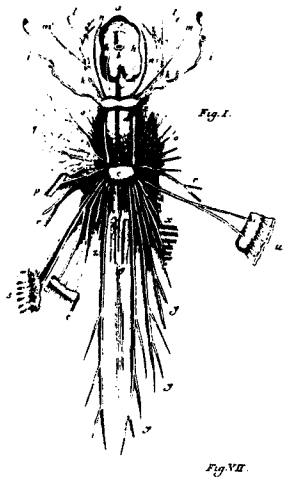
Download date: 05. May. 2023

a
4594
ZD

RESPIRATORY BEHAVIOUR OF THE PULMONATE FRESHWATER SNAIL *LYMNAEA STAGNALIS* AND ITS NEURAL CONTROL

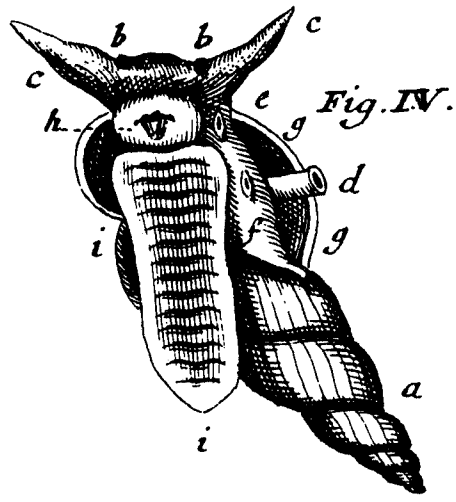
Gertjan van der Wilt

TAB VI.



Wyn gaart-Slak.

Herſenen en Senuen.



Gemeene Water-Slak.

a 045941.2D

STELLINGEN

behorend bij het proefschrift van Gertjan van der Wilt,
Respiratory behaviour of the pulmonate fresh water snail Lymnaea stagnalis and its neural control

Opheldering van de betekenis van sterk divergerende synaptische inputs in het centraal zenuwstelsel van *Lymnaea* zou in belangrijke mate kunnen bijdragen aan ons begrip van de ontwikkeling en het funktioneren van het zenuwstelsel.

Membraan gebonden eiwitten met eigenschappen van ademhalingspigmenten kunnen in principe een functie vervullen bij het transductieproces van O_2 chemosensorische informatie.

In moderne concepten van de organisatie van neuronale netwerken heeft een strakke hiërarchische structuur plaats gemaakt voor reciprociteit, waarin functies toegeschreven worden aan interacterende cellen i. p. v. aan enkelvoudige cellen, en beslissingen niet berusten bij enkelvoudige commandocellen maar consensus weerspiegelen tussen meerdere cellen. De aard van de conceptuele veranderingen en de termen die gebezigd worden suggereren dat deze concepten sterk beinvloed worden door maatschappelijke ontwikkelingen.

Systematisch onderzoek naar verschillen tussen simpele proefdieren enerzijds en hoger ontwikkelde organismen anderzijds kan minstens zo informatief zijn als systematisch onderzoek naar overeenkomsten tussen beiden.

Fundamenteel onderzoek naar de verspreiding van slakken kan belangrijke toepassingswaarde hebben. Lovell, M. S. The edible Molluscs of Great Brittain and Ireland with recipies for cooking them. London, 1867.

Het feit dat de Britse onderzoeker Jones verschillen in onderzoeksresultaten toeschrijft aan het feit dat hij, in tegenstelling tot Wolvekamp en Zaaijer, Britse slakken gebruikt, illustreert dat het besef dat Britten anders zijn, diep geworteld is. Jones, 1961.

De mogelijkheid om in de medische wetenschap artikelen te publiceren die gebaseerd zijn op de bevindingen bij één of enkele patiënten is niet bevordelijk voor een evenwichtige wetenschapsbeoefening.

Het feit dat 80 % van de Nederlandse bevolking voorstander is van het gebruik van organen van overledenen voor transplantatie doeleinden, terwijl slechts 20 % drager is van een donor codicil, hoeft op zich nog geen aanleiding te zijn om in Nederland over te gaan op het "geen bezwaar systeem".

Ten onrechte wordt snelle veroudering van wetenschappelijke onderzoeksresultaten veelal beschouwd als teken van een gunstige ontwikkeling van het betreffende onderzoeksgebied.

Het toestaan door de overheid van tabaksreclame maakt het gehele volksgezondheidbeleid volstrekt ongeloofwaardig.

De stelselwijziging (het zg. 'plan Simons') houdt in alle opzichten een verbetering in ten opzichte van het huidige gezondheidszorgstelsel.

Gertjan van der Wilt, 18 november 1992

a.045942D

RESPIRATORY BEHAVIOUR OF THE PULMONATE FRESHWATER SNAIL *LYMNAEA STAGNALIS* AND ITS NEURAL CONTROL

Gertjan van der Wilt

VRIJE UNIVERSITEIT

RESPIRATORY BEHAVIOUR OF THE PULMONATE FRESHWATER SNAIL *LYMNAEA STAGNALIS* AND ITS NEURAL CONTROL

ACADEMISCH PROEFSCHRIFT

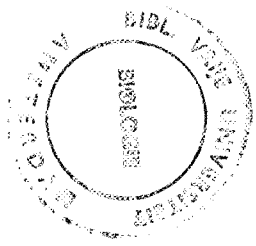
ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
dr. C. Datema,
hoogleraar aan de faculteit der letteren,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der biologie
op woensdag 18 november 1992 te 10.30 uur
in het hoofdgebouw van de universiteit, De Boelelaan 1105

door

Gerrit Jan van der Wilt
geboren te Accra (Ghana, West Afrika)

Centrale Huisdrukkerij Vrije Universiteit
Amsterdam
1992

Promotor:	prof.dr. Th.A. de Vlieger
Copromotor:	dr. C. Janse
Referent:	prof.dr. ir. L. M. Schoonhoven



Ik had dit boekje graag eens aan mijn vader laten zien

typing: Thea Laan

photography: Joop Meijer and his colleagues

drawings: Luigi Sanno

cover: Drawings by the Dutch biologist Jan Swammerdam (1637 - 1680) of the central nervous system of the Roman snail *Helix* (left), and of the pond snail *Lymnaea* (right) showing the respiratory organ (d). The drawings are part of his *Biblia Naturae* (The Bible of Nature), which appeared after his death in 1737 - 1738 at Isaak Severinus in Leyden, thanks to the efforts of Herman Boerhaave. A facsimile edition of the *Biblia Naturae* was prepared by De Banier (Utrecht) and De Groot (Goudriaan). By the courtesy of Florence Pieters of the University of Amsterdam library at *Artis Natura Magister*.

The research described in this thesis was carried out at the Department of Neurophysiology of the Faculty of Biology, Vrije Universiteit in Amsterdam. The research was supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Scientific Research (NWO).

CONTENTS

Chapter 1 Inleiding en samenvatting (Introduction and summary, in Dutch) 13
1. NEUROFYSIOLOGIE: DE VRAGEN EN DE ANTWOORDEN 14
2. NEUROWETENSCHAP IN AKTIE 17
2.1. Is de werking van een neuronaal netwerk te begrijpen?
2.2. Modellen van neuronale netwerken: verschillende interpretaties
2.3. Minimale eisen voor een goed begrip van de werking van een neuronaal netwerk
2.4. Stabiele patronen versus flexibiliteit
2.5. Netwerkfunktie en variabiliteit in het netwerk: redundantie
2.6. Geen algemene principes?
3. HET ADEMHALINGSGEDRAG VAN *LYMNAEA STAGNALIS* EN DE NEURONALE BASIS 33
3.1. Bimodale gasuitwisseling
3.2. Anaërobe stofwisseling
3.3. Fluctuaties in bloed-zuurstofgehalte
3.4. Faktoren die van invloed zijn op longademhaling
3.5. Ademhaling: interferentie met andere typen van gedrag
3.6. Het *hoe* en *waarom* van het ademhalingsgedrag van *Lymnaea:* evolutietheorie en neuronaal netwerkanalyse
3.7. Relatie met overig onderzoek binnen de Vakgroep Organismale Dierkunde
3.8. Nogmaals: Neurowetenschap in aktie

Chapter 2 Multimodal sensory control of respiratory behaviour in the pulmonate freshwater snail *Lymnaea stagnalis* (Mollusca, Gastropoda) 43

Chapter 3 Relationship between haemolymphPO_2, respiratory behaviour, heart rate and anaerobic metabolism, in the freshwater pulmonate snail *Lymnaea stagnalis* 63

Chapter 4 Suppression of respiratory behaviour during egg laying in the freshwater pulmonate snail *Lymnaea stagnalis* 80

Chapter 5 Interaction between respiratory and feeding behaviour in the freshwater pulmonate snail *Lymnaea stagnalis* 89

Chapter 6 Central and peripheral neurones involved in oxygen reception in the pulmonate snail *Lymnaea stagnalis* (Mollusca, Gastropoda) 99

Chapter 7 Neural control of pneumostome movements in the freshwater pulmonate snail, *Lymnaea stagnalis* 117

Chapter 8 Effects of histamine and catecholamines on electrical activity of peptidergic neurons, involved in control of respiration in the freshwater snail *Lymnaea stagnalis* 151

DANKWOORD

In elk proefschrift worden allerlei mensen bedankt die geholpen hebben bij de totstandkoming ervan. Pas nu ik zelf zo ver ben om het dankwoord te schrijven, besef ik de betekenis pas goed. Laat ik in min of meer chronologische volgorde een aantal mensen noemen die mij in de afgelopen jaren enorm hebben geholpen.

Om te beginnen was er tijdens de studie en in alle jaren die volgden Nico de With; jij was een voorbeeld als het ging om zorgvuldig onderzoek en werkelijk nadenken over de betekenis van je onderzoeksresultaten. Door jou werd biologie *echt* een leuk vak. Hiervoor en voor de vriendschap door de jaren heen, ben ik je zeer dankbaar. In diezelfde periode kon ik altijd terugvallen op de deskundige en vriendschappelijke assistentie van Roel van der Schors. En dan was er natuurlijk Joos Joosse. Jij zorgde ervoor dat ik indertijd mee kon naar een congres over biomineralisatie in Zeeland. Volgens mij heb je het nog betaald ook. Het is me altijd een raadsel gebleven hoe het mogelijk was dat je altijd tijd had: tijd voor een praatje over werk of anderszins. Je stimulerende enthousiasme en je vriendschap zijn heel belangrijk voor me geweest.

Vervolgens was er een stage bij Neurofysiologie. Cees Janse - jij hebt mij ingewijd in de wereld van de neurowetenschappen. Je hebt me indertijd begeleid tijdens mijn stage en je hebt het initiatief genomen om een subsidieaanvraag bij NWO in te dienen voor een vervolgonderzoek. Deze aanvraag werd toegewezen en je hebt de dagelijkse begeleiding van dit onderzoek op je genomen. Dat heeft het mij mogelijk gemaakt om verder te gaan in de neurowetenschappen en ik ben je daar zeer dankbaar voor. Je hebt me gestimuleerd en het mogelijk gemaakt om de onderzoeksresultaten in binnen- en buitenland te presenteren. Ik bewaar goede herinneringen aan Budapest, Tihany, Marseille, Zürich, Bangor etc De eerste presentatie was volgens mij in Utrecht voor de Nederlandse Dierkundige Vereniging. Wat ging daar een boel mis!

Jean Feyen en Peter Nijweide. Zij verleenden me gastvrijheid op het laboratorium voor Celbiologie en Histologie van de Medische faculteit in Leiden. Jean, het was een groot plezier om met je samen te kunnen werken en ook van jou kon ik veel leren (let wel, ik zeg niet dat dat ook werkelijk is gebeurd!).

Vervolgens was er Tom Piek, die me gastvrijheid verleende op het Farmacologisch Laboratorium van de Medische Faculteit van de UvA. In een heel vroeg stadium mocht ik daar ervaring opdoen met de patch-clamptechniek.

Tijdens mijn promotieonderzoek en het schrijven van mijn proefschrift hebben nog vele anderen me bijgestaan.

Andries ter Maat; je hebt me enorm geholpen bij de analyse en interpretatie van de resultaten van het gedragsonderzoek. Ik realiseer me dat je er veel tijd in hebt gestoken. Ik waardeer dat des te meer omdat je formeel geen verantwoordelijkheid draagt voor mijn onderzoek. Het contact met jou en Hanneke, de avonturen met Graham en met Laura en Lorenzo - toch een beetje 'de jus' van het werk?

Theo Wijsman; het was een plezier om met jou samen onderzoek te doen naar anaëroob metabolisme. Dank voor je deskundige begeleiding hierbij.

Jacques Bedaux; jij hebt me heel wat geholpen bij de statistische verwerking van m'n onderzoeksgegevens. Wat denk je, paarlen voor de zwijnen?

Marcel van der Roest; tijdens de hele periode heb je me uitstekend geholpen bij de uitvoering van het onderzoek en je hebt zelfstandig heel goed onderzoek gedaan. Je hulp bij het vele 'plak-en-knip werk' op het laatst heb ik ook erg gewaardeerd. Zeer bedankt!

Wic Wildering; ik vond het erg leuk om met je samen te werken. Het was ook interessant om eens van een ander te horen hoe hij het promotieonderzoek ervaart. Onze tocht door Zwitserland zal ik niet snel vergeten.

Hans Lodder; tja, hoe zeg je dat? Je straalt iets uit van 'het komt allemaal wel goed.' Die uitstraling komt wel eens van pas. Het grappige is: je maakt het nog waar ook.

Mart Mojet. Als doctoraal-student heb je op heel zelfstandige wijze een flinke bijdrage aan het onderzoek geleverd. Het zou me niet verbazen wanneer veel van je ideeën over regulatie van ademhaling, hartslag en locomotie, en de rol van VD1 en RPD2 daarbij, bewaarheid zullen worden. Je hebt veel nieuwe dingen gedaan; je hebt bovendien hard gewerkt.

Theo de Vlieger. Zonder jou was het natuurlijk allemaal niks geworden. Op een kritisch moment wist je het geheel ten goede te keren. Hoe zei je het ook al weer? Bij tegenwind gaat een vlieger juist omhoog? Tenslotte: een gemeenschappelijke waardering voor Jacques Tati spreekt volgens mij boekdelen.

Prof. dr. ir. L. M. Schoonhoven - ik ben U zeer dankbaar dat U bereid was om als referent bij dit proefschrift op te treden. Ik bewaar goede herinneringen aan de dag dat Theo de Vlieger en ik naar Uw lab in Wageningen gingen om het manuscript aan te bieden.

Eric Jan Tuininga en Bert Musschenga, mijn huidige werkgevers: jullie hebben mij een verlof toegestaan om het proefschrift af te ronden.

Wim van der Steen, voor zijn bereidheid om een eerdere versie van de inleiding te lezen en te voorzien van nuttig commentaar.

Verder zeer veel technische ondersteuning van:

- de foto- en tekenkamer: Joop Meijer en z'n medewerkers en Luigi Sanno. Altijd vriendelijk en fantastische kwaliteit. Zeer bedankt!

- het secretariaat, vooral Thea Laan. Hartelijk dank voor de zeer vakkundige assistentie bij het typewerk en de layout
- de instrumentmakerij onder leiding van de heer Valkier; met name dank aan Chiel Ehrenfeldt en Rob Stoevelaar, voor ingebruikstelling en onderhoud van de gasflow-apparatuur
- de elektronica-afdeling van Martien van Vilsteren en zijn medewerkers, dank voor het ontwerpen en vervaardigen van een infra-rood sensor en voor de constante ondersteuning
- de dierverzorging; Carool Popelier en Sara Anastacio die zorgden voor een continu aanbod van slakken. Bestellingen leverden nooit problemen, gebruik van de doorstroombakken was altijd mogelijk
- en tenslotte natuurlijk ondersteuning van de kant van de bibliotheek en de administratie
Al deze bereidheid tot medewerking, ondervonden in de afgelopen jaren, hebben bijgedragen aan een prima werksfeer.

Daarnaast waren er natuurlijk familie -in de eerste plaats mijn moeder en broer- en vrienden die meeleefden en zich afvroegen hoe het nu zat met dat proefschrift. Eduard Kimman die mij nu al drie jaar overal introduceert als 'die jongen die op het punt staat te promoveren in de neurowetenschappen'. En, *last but not least* mijn echtgenote, Isa, die zich af en toe hoofdschuddend afvroeg of het dat nu wel allemaal waard was; wat denk je - nu jij? Ciao!

Gertjan van der Wilt, Amsterdam 13 september 1992.

CHAPTER 1

INLEIDING EN SAMENVATTING

'..science is always on the move. Its preference is to find a question that nobody knew needed answering, answer it and then move on, leaving technologists to turn the answer into a machine, a drug or a computer program.'
Matt Ridley, The Economist, 16 februari 1991.

Bovenstaand citaat suggereert dat voor veel vragen die aan wetenschappelijk onderzoek ten grondslag liggen geldt dat niemand, behalve de onderzoekers zelf, wist dat het om vragen ging die beantwoord dienden te worden. Dit geldt ook voor het onderzoek dat beschreven wordt in dit proefschrift. Als U het leest komt U iets te weten over het ademhalingsgedrag van een zoetwaterslak, en over de zenuwcellen die betrokken zijn bij de regulatie van dat gedrag. Het onderzoek is een poging om een relatie te leggen tussen het gedrag van een dier enerzijds en de aktiviteit van zenuwcellen in de hersenen van dat dier anderzijds. Het doel van dergelijk onderzoek is, vast te stellen hoe die relatie er precies uitziet: hoe correleren aspecten die waargenomen kunnen worden aan het gedrag van het dier met gebeurtenissen die met behulp van bepaalde technieken waargenomen kunnen worden in de hersenen van dat dier? Voor velen zal dit waarschijnlijk niet een kwestie zijn van 'everything you always wanted to know about ...'. In de hoop toch iets over te brengen van wat een neurofysioloog beweegt, zal ik mij in deze inleiding niet beperken tot een toelichting van de antwoorden, maar ook stilstaan bij de vragen die aan het onderzoek ten grondslag liggen.

Mijn uitgangspunt voor deze inleiding zal zijn dat er iets zichtbaar gemaakt moet worden van de wijze waarop de vragen in een wetenschap tot stand komen, wil deze wetenschap enigszins begrijpelijk zijn voor een buitenstaander. Een wetenschappelijke theorie die probeert bepaalde verschijnselen te verklaren veronderstelt dat het de moeite waard is om deze verschijnselen te verklaren. Dit lijkt misschien triviaal, maar dat is het naar mijn idee niet. Niet alle verschijnselen zijn de moeite waard. Dat kan verschillende redenen hebben. Bijvoorbeeld dat men van mening is dat bepaalde verschijnselen voor zich zelf spreken, en dus geen verklaring behoeven. Of dat een verschijnsel weliswaar niet te verklaren is, maar dat een verklaring op korte termijn niet te verwachten is. Ik zal in deze inleiding proberen te illustreren, niet alleen *wat* men probeert te verklaren m. b. v. neurofysiologisch onderzoek, maar ook *waarom* bepaalde dingen de moeite waard gevonden worden om te onderzoeken, wat acceptabele methoden zijn van onderzoek en

wat aannemelijke verklaringen voor de onderzochte verschijnselen zijn. Voor dit doel zal ik een discussie weergeven tussen neurofysiologen over de vraag of de werking van netwerken van zenuwcellen überhaupt te begrijpen is. Ik heb hiervoor gekozen omdat naar mijn mening in deze discussie zichtbaar wordt welke argumenten een rol spelen bij vragen als: Wat is nu wel, wat is nu niet de moeite van het onderzoeken waard? Wat zijn geldige onderzoeksmethoden? Wat zijn aannemelijke verklaringen voor de werking van netwerken van zenuwcellen? Welke gegevens zijn noodzakelijk -en tevens voldoende- willen we het idee hebben dat we de werking van een netwerk van zenuwcellen werkelijk begrijpen? Welk type onderzoek, welke gegevens zijn nodig om aannemelijk te maken dat een bepaald gedrag verklaard kan worden vanuit de eigenschappen van zenuwcellen en hun onderlinge verbindingen? Er zal blijken dat op deze vragen geen eenduidig antwoord wordt gegeven. Ik heb de betreffende paragraaf de titel 'Neurowetenschap in aktie' gegeven, ontleend aan de titel van het boek van Bruno Latour *Wetenschap in aktie* (1988), over ontwikkeling van wetenschap. Voordat ik hier verder op doorga volgt hieronder eerst een wat algemenere inleiding op neurofysiologisch onderzoek.

1. NEUROFYSIOLOGIE: DE VRAGEN EN DE ANTWOORDEN

Voor een neurofysioloog kan het gedrag van een dier makkelijk aanleiding geven tot het formuleren van vragen. Een dier zal bijvoorbeeld op sommige signalen uit z'n omgeving reageren met een bepaald gedrag, terwijl het andere signalen negeert. Hoe is het dier in staat om relevante signalen uit de omgeving waar te nemen, en deze te onderscheiden van niet-relevante signalen? En waarom reageert het dier op deze signalen op deze specifieke manier, en niet anders? We kunnen bijvoorbeeld ook vaststellen dat een dier niet altijd op dezelfde wijze reageert op signalen uit z'n omgeving. Op basis van welke faktoren besluit een dier of en hoe hij zal reageren? [1] Op deze vragen kunnen verschillende soorten antwoorden gegeven worden: het gedrag kan op verschillende manieren verklaard worden.

Een voorbeeld: kat en muis

Als we een muis heel hard zien wegrennen, wat zou dan een adequate verklaring van dat gedrag kunnen zijn? We zouden kunnen beginnen met vast te stellen dat er een kat achter de muis aan zit. We kunnen dit een afdoende verklaring vinden; we kunnen ook vaststellen dat dit een afdoende verklaring is omdat deze gebaseerd is op een heel aantal

[1] Camhi (1984) spreekt in dit verband van: 'detection of biologically significant signals, recognition, discrimination of these signals from background noise, localization of sensory cues, the decision by an animal whether or not to respond to a given sensory stimulus by producing a behavioral act, orientation and the control of complex behavioural acts (mechanisms by which different parts of the body are integrated into a coordinated movement), learning etc.

vooronderstellingen die we allemaal aannemelijk vinden. Eén vooronderstelling is bijvoorbeeld dat de muis de kat heeft gezien, en denkt dat die hem op wil eten. Bovendien denkt de muis dat hard wegrennen de beste manier is om aan de kat te ontsnappen. Aan de kat kunnen we soortgelijke eigenschappen toeschrijven.

Daarnaast kunnen we verwijzen naar de morfologie en de fysiologie van de kat en de muis: beiden hebben kennelijk de zintuigen om elkaar waar te nemen en het vermogen om de waarneming te interpreteren (gevaar! of: lekker hapje!) en te beslissen wat de beste reaktie is in dit soort situaties (rennen!). Bovendien beschikken beide dieren over een skelet, spieren en een zenuwstelsel, waardoor ze kunnen rennen. Aan spiercellen en aan zintuig- en zenuwcellen van het oog kunnen we ook weer bepaalde eigenschappen toeschrijven: eigenschappen van zintuigcellen waardoor deze licht kunnen omzetten in electrische signalen, eigenschappen van zenuwcellen waardoor deze selectief reageren op bepaalde patronen, eigenschappen van zenuwcellen waardoor ze met elkaar kunnen communiceren via electrische signalen etc.

We zouden nog lagere niveaus van organisatie kunnen kiezen voor een verklaring van het gedrag dat we hebben waargenomen, bv. bindingseigenschappen van receptoren in het post-synaptische membraan voor bepaalde transmitters, conformatieveranderingen van eiwitten (bv. ionkanalen) in het membraan van een zenuwcel als gevolg van electrische spannigsveranderingen over het membraan etc.
Een heel ander soort verklaringen is mogelijk, namelijk dat we naar de evolutie van soorten moeten grijpen om te begrijpen waarom katten muizen vangen (en over alle eigenschappen beschikken die hen dit mogelijk maken) en waarom muizen bang zijn voor katten (en over alle eigenschappen beschikken om uit de klauwen van katten te blijven). Dit kan eventueel nog aangevuld worden met een verklaring die verwijst naar de ontwikkeling van het individuele dier, en naar de persoonlijke levensgeschiedenis van de kat en de muis: ervaringen die beiden met de ontmoeting van de ander hebben opgedaan.

We zouden tenslotte ook op zoek kunnen gaan naar een verklaring waarom er eigenlijk katten zijn, waarom er muizen zijn, en waarom de één achter de ander aan zit; wat is de zin van dat alles?

Gegeven deze verscheidenheid aan mogelijke verklaringen van dierlijk gedrag, doet zich de vraag voor: is de ene verklaring beter dan de andere? Of vullen ze elkaar aan? Is er een relatie tussen de verschillende verklaringen, en zo ja, welke? Is de angst van de muis voor de kat te verklaren vanuit de eigenschappen van een aantal zenuwcellen? En: wat is de relatie tussen de angst van de muis en de afgifte van stoffen in de hersenen van het dier? Is het rennen van de kat te verklaren met behulp van eigenschappen van zenuwcellen?

Dergelijke vragen vormen van tijd tot tijd de inzet van een discussie tussen neurowetenschappers: Welke aspecten van het gedrag - de muis die wegrent voor de kat -

zijn de moeite waard om te onderzoeken? Hoe moeten we dat doen, en wanneer beschikken we over een adequate verklaring voor de waargenomen verschijnselen?

Het hoe en waarom

In de neurowetenschappen kunnen de vragen die gesteld worden naar aanleiding van waarnemingen aan het gedrag van dieren in twee categorieën verdelen: hoe-vragen, en waarom-vragen. Antwoorden op 'hoe vragen' moeten duidelijk maken welke eigenschappen van een dier het in staat stellen om een bepaald soort gedrag te vertonen. De belangrijkste vooronderstelling die hierbij gemaakt wordt, is dat elke vorm van gedrag een materiële basis heeft in de vorm van fysisch-chemische gebeurtenissen in het zenuwstelsel. Gedrag moet in dit verband ruim opgevat worden: hieronder vallen bijvoorbeeld voortbewegen (lopen, zwemmen, vliegen etc) en eten, maar ook funkties als hartslag en ademhaling, en hogere hersenfunkties als de cognitieve funkties en emoties. De chemisch-fysische processen zijn hoofzakelijk electrische verschijnselen die zich afspelen in zenuwcellen. De vooronderstelling dat alle aspecten van het gedrag van een dier een weerspiegeling zijn van de aktiviteit van zenuwcellen is weliswaar niet helemaal onomstreden, maar ze is evenmin onderwerp van expliciete toetsing. Camhi (1984) geeft voor mijn gevoel vrij goed weer hoe onder neurofysiologen de houding is ten opzichte van de stelling dat gedrag volledig verklaarbaar in termen van structuur en funktie van het zenuwstelsel:

> *Physicochemical factors play a role in behavior, and neuroscientists try to understand these factors. Although it cannot be absolutely proved that such factors will fully explain all the behavior of animals, scientists simply have no adequate means to study any non-physical forces that may exist. Therefore, most scientists have stopped trying.*

Antwoorden op 'waarom vragen' moeten duidelijk maken waarom een dier zich op een bepaalde manier gedraagt, problemen op een bepaalde manier oplost, en niet anders. Voor een antwoord op deze vraag nemen we onze toevlucht tot de evolutietheorie. Omdat het gedrag van dieren voor een deel genetisch vastgelegd is, is het ook onderhevig aan de processen van variatie en selectie. Als gevolg van deze processen veronderstellen we dat een dier optimaal is aangepast aan z'n omgeving; we schrijven aan gedrag een bepaalde betekenis toe door het in verband te brengen met funkties als eten, drinken, voortplanting, vluchten etc. Het gedrag van dieren wordt op deze manier geanalyseerd door er betekenis aan toe te kennen voor de overleving van het dier.

Tot zover heb ik geprobeerd duidelijk te maken dat waarnemingen aan het gedrag van dieren aanleiding kunnen geven tot het stellen van verschillende soorten vragen, en dat voor elk van de vragen verschillende antwoorden mogelijk zijn. Welke vragen relevant

zijn, en welke antwoorden adequaat, vormt de inzet van een discussie tussen wetenschappers. In het volgende deel geef ik een dergelijke discussie weer tussen neurowetenschappers.

2. NEUROWETENSCHAP IN AKTIE

2.1. Is de werking van een neuronaal netwerk te begrijpen?

In een artikel in The Behavioral- and Brain Sciences stelt de neurowetenschapper Allen Selverston de vraag of bepaalde netwerken van zenuwcellen -centrale patroon-generatoren- te begrijpen zijn (Selverston, 1980). In het volgende zal ik dit artikel samenvatten, en uitleggen wat een centrale patroon generator is en wat Selverston verstaat onder 'het begrijpen' van de werking van zo'n netwerk.

2. 1. 1. *Het concept van de Centrale - Patroongenerator*

Sommige gedragingen van dieren bestaan uit een bepaald patroon van spiercontracties, dat zich vootdurend op min of meer dezelfde manier herhaalt. Voorbeelden hiervan zijn voortbeweging van dieren (lopen, vliegen, zwemmen), kauwbewegingen, ademhaling en hartslag. Hoewel variaties kunnen optreden, is het basispatroon tamelijk constant. Voor een aantal gevallen is aangetoond dat dergelijke ritmische spierbewegingen tot stand komen als gevolg van electrische akitiviteit van een groep van zenuwcellen die zelfstandig - dat is, zonder input van buitenaf - in staat zijn een heel specifiek patroon van electrische aktiviteit tot stand te brengen. De zenuwcellen maken onderling contact, en ze maken contact met specifieke spiercellen; electrische aktiviteit in de zenuwcellen (motorneuronen) heeft contractie van de spiercellen tot gevolg. Vanwege het relatief stabiele patroon van spiercontracties mag verwacht worden dat het patroon van electrische aktiviteit van motorneuronen dat verantwoordelijk is voor deze spiercontracties ook relatief stabiel zal zijn: dezelfde groep van motorneuronen zal, wanneer het gedrag zich voordoet, steeds weer min of meer hetzelfde patroon van electrische aktiviteit genereren. Een dergelijke groep van zenuwcellen, die met elkaar in staat zijn om een patroon van ritmische aktiviteit te genereren, wordt een centrale patroongenerator genoemd (CPG).

Een CPG kan bestaan uit uitsluitend motorneuronen (zenuwcellen die rechtstreeks contact maken met de spieren), uit pre-motorneuronen (interneuronen, die via speciale contacten - zg. synapsen - contact maken met motorneuronen, die op hun beurt de spieren innerveren), of beide.

Samenvattend: geordende spierbewegingen kunnen het gevolg zijn van patronen van electrische aktiviteit die ontstaan in netwerken van zenuwcellen.

Dat CPG' s zelfstandig in staat zijn om een geordend patroon van electrische aktiviteit tot stand te brengen blijkt uit het feit dat ze kunnen funktioneren in een geïsoleerd

zenuwstelsel. Wanneer de CPG één keer geaktiveerd is kan deze voortgaan met het produceren van een karakteristiek patroon van aktiviteit, zonder dat daar input van buitenaf (bv. de spieren) voor nodig is.

De vraag die Selverston nu stelt is: hoe werkt zo' n CPG? Kunnen we dat begrijpen? Welke eigenschappen hebben neuronen, en welke eigenschappen hebben netwerken van neuronen, waardoor ze in staat zijn een dergelijk patroon van aktiviteit het genereren, dat aanleiding geeft tot een gecoördineerd patroon van spierbewegingen die deel uitmaken van een bepaald gedrag?

2. 1. 2. *Onderzoeksstrategie*

Wat voor onderzoek moeten we doen, en over welke gegevens moeten wij minimaal beschikken, opdat we, zo vraagt Selverston zich af, om de werking van zo' n netwerk van zenuwcellen (CPG) te kunnen begrijpen? Hij stelt de volgende strategie voor: 1) identificatie en karakterisering van alle componenten (zenuwcellen en hun onderlinge verbindingen) die deel uitmaken van het netwerk (de CPG), 2) verklaring van de ritmische output vanuit de eigenschappen van de verschillende componenten (zenuwcellen) en de verbindingen tussen de zenuwcellen onderling, en 3) op basis van deze gegevens ontwikkeling van een model van de CPG, dat niet alleen in staat is om dezelfde output te genereren, maar bovendien kan voorspellen wat het effect is van verandering van de aktiviteit van één of meer van de componenten van het netwerk op de output van het systeem. Dit laatste kan bijvoorbeeld een computersimulatie van het netwerk zijn.

Uit het voorgaande volgt dat we eerst over criteria moeten beschikken om te bepalen of een zenuwcel al of niet deel uitmaakt van een bepaald netwerk (CPG). Selverston noemt als criterium *correlatie*: is er sprake van correlatie tussen de aktiviteit van een zenuwcel en de ouput van de CPG? Als dat het geval is, dan moeten we vervolgens vaststellen wat de rol van die cel in het netwerk is: wat gebeurt er met de aktiviteit van het netwerk als geheel wanneer we een cel funktioneel uit het netwerk onttrekken? Daarnaast hebben we criteria nodig aan de hand waarvan we kunnen vaststellen of een zenuwcel een motorneuron is of een interneuron: maakt de zenuwcel direct contact met een spiercel of niet? Selverston noemt een aantal electrofysiologische technieken die uitsluitsel moeten geven over het voorgaande, waar ik hier verder niet op in zal gaan [2].

---

[2] Het gaat om criteria als correlatie met de motor output, resetting experimenten; effecten van depolarizatie, hyperpolarizatie, fotodynamische inactivatie etc op de output van de CPG; 1 : 1 correlatie tussen aktiepotentialen in het motorneuron en potentialen in de spier; backfilling van de zenuw moet in principe alle motorneuronen aankleuren. Deze criteria zijn elk op te vatten als *operationele definities* van een aantal belangrijke begrippen. Met een operationele definitie bedoel ik het volgende: om vast te stellen of een cel deel uitmaakt van een netwerk, een motorneuron is e. d. moet een bepaalde experimentele handeling vericht worden; aan de hand van de uitkomst van deze handeling kan vastgesteld worden of een cel aan bepaalde criteria voldoet, zodat deze al of niet aangemerkt kan worden als element in een netwerk, als motorneuron etc.

2. 1. 3. *De 'minimal list'*

Op basis van de voorgaande overwegingen stelt Selverston een lijst van criteria samen waar een analyse van een netwerk minimaal aan moet voldoen, willen we het funktioneren van het betreffende netwerk kunnen begrijpen. Deze lijst ziet er als volgt uit:

- we moeten alle componenten (dat wil zeggen: cellen) van het netwerk kennen. Hierbij merkt hij meteen op dat dit onmogelijk is met de huidige technieken; je kunt er nooit zeker van zijn dat je geen cellen hebt gemist.
- van alle componenten moeten de individuele eigenschappen bekend zijn. Bijvoorbeeld: hoe vuurt de cel (regelmatig, onregelmatig, in bursts etc), is de cel makkelijk te exciteren of juist niet, wat zijn de endogene eigenschappen van de cel (dat wil zeggen: hoe gedraagt de cel zich wanneer je hem volledig isoleert van alle andere cellen, bv. is een cel een endogene burster). Bovendien moeten we vaststellen of deze eigenschappen afhankelijk zijn van het aktiviteitsniveau van het netwerk (de zg. dynamische eigenschappen van het netwerk)
- hoe zijn de verbindingen tussen de verschillende componenten van het netwerk onderling; dat wil zeggen: welke cellen maken synaptische contacten met elkaar, en wat is de aard van die contacten? Zijn deze contacten monosynaptisch? In welke mate is de ene cel in staat de aktiviteit van de andere cel te beïnvloeden?

Dit is de 'minimal list': deze gegevens zijn noodzakelijk om de werking van een netwerk te kunnen begrijpen. De vraag is: zijn deze gegevens ook voldoende om ze te kunnen begrijpen?

2. 1. 4. *Modelleren van een netwerk*

Het resultaat van het experimentele werk is dat we een bepaald patroon van electrische aktiviteit hebben beschreven, we hebben een aantal cellen beschreven waarvan de aktiviteit een relatie vertoont met dit patroon, de eigenschappen van die cellen, en hun onderlinge contacten. Nu zal, aldus Selverston, een complete beschrijving van een neuronaal netwerk (één die aan bovenstaande criteria voldoet) nog niet voldoende zijn om te begrijpen hoe het netwerk funktioneert. Wat hij hier volgens mij bedoelt, is dat we gewoonweg niet in staat zijn om een ingewikkeld schema van onderling verbonden neuronen, met specificaties van individuele eigenschappen en eigenschappen van synaptische contacten, te 'lezen', op een zodanige manier dat we kunnen nagaan of dat specifieke netwerk ook in staat is om een bepaalde aktiviteit te genereren, en alle overige waarnemingen aan het netwerk te verklaren (we kunnen die informatie niet op die manier verwerken). Voor heel simpele netwerken, die bestaan uit een heel klein aantal cellen (bv. 2 - 4) en een klein aantal onderlinge verbindingen, bij voorkeur van één soort, is dat nog te doen; wanneer een netwerk uit grotere aantallen cellen bestaat met talrijke onderlinge verbindingen, is dat niet of nauwelijks meer te doen. Wat we dan nodig hebben is een

model (bv. een computersimulatie); we bouwen het netwerk als het ware na. De elementen van zo' n model zijn de zenuwcellen en hun ondelinge verbindingen, waar we bepaalde eigenschappen aan toe schrijven op basis van experimenteel verkregen gegevens. De vraag is dan: is het model in staat patronen van aktiviteit te genereren die waargenomen zijn aan het eigenlijke netwerk? Het nut van dergelijke modellen is dat we configuraties van cellen met hun onderlinge contacten op het spoor kunnen komen die verantwoordelijk *zouden kunnen* zijn voor het genereren van ritmische aktiviteit in een neuronaal netwerk. Anderzijds, wanneer configuraties niet in staat zijn de waargenomen verschijnselen na te bootsen, kunnen we deze verwerpen.

2. 1. 5. *Ritmische aktiviteit: eigenschappen van cellen of van een netwerk als geheel?*
Selverston maakt een onderscheid tussen twee fundamenteel verschillende typen van model: 1) een model waarbij een neuronaal netwerk gedreven wordt door één of meer cellen die spontaan ritmische aktiviteit vertonen (zg. endogene bursters), en 2) modellen waarbij geen van de cellen van het netwerk een dergelijke eigenschap heeft, maar waarbij ritmische aktiviteit van het netwerk een gevolg is van specifieke schakelingen tussen de cellen ('emergent property').

Wanneer een cel volledig is geïsoleerd van alle input die hij normaal ontvangt, en vervolgens ritmische aktiviteit vertoont, dan is dit voldoende aanwijzing dat de cel een endogene burster is. Voor het geval van ritmiciteit als netwerkeigenschap bespreekt Selverston drie mogelijke schakelingen die dit kunnen veroorzaken: reciproke inhibitie, inhibitoire loops, en excitatoire netwerken. Dit zijn theoretische modellen van schakelingen tussen neuronen, die in principe in staat zijn om cyclische aktiviteit te genereren; er zijn experimentele gegevens die er op wijzen dat in sommige neuronale netwerken dergelijke mechanismen daadwerkelijk voorkomen.

2. 1. 6. *Nut van modelleren*
Selverston hinkt hier echter op twee gedachten: enerzijds ziet hij de noodzaak van modelleren wel in, anderzijds is hij echter van mening dat het nut van dergelijke modellen sterk overschat wordt. Hij noemt een aantal voorbeelden waarbij een neuronaal netwerk tot op zekere hoogte is geanalyseerd, en de gegevens werden gebruikt om een model van het netwerk te ontwerpen. Vervolgens bleek dit model inderdaad de waargenomen verschijnselen te kunnen verklaren, en werd aangenomen dat het model een goede beschrijving was van het netwerk. In daarop volgende jaren bleek echter uit vervolgonderzoek dat de oorspronkelijke experimentele gegevens onjuist, of onvolledig waren. De nieuwe gegevens waren niet verenigbaar met het model, dat verworpen moest worden. Welke les kunnen we hier uit trekken volgens Selverston? Dat een model eigenlijk pas gebruikt moet worden ter verificatie of falsificatie van een bepaalde

configuratie van een netwerk, wanneer we *alle* gegevens van een netwerk, opgesomd in de 'minimal list' tot onze beschikking hebben. Letterlijk zegt hij:

> *Supplied with insufficient, often incorrect information, the model will usually have enough variable parameters for the modeler to produce any rhythm he desires for any circuit he sets out to model. Modeling has its place. When the totality of a circuit (= CPG) is established, with all the monosynaptic connections and all the elemental properties of the cells and synapses known, then a model should be able not only to replicate the motor output pattern but also to accurately predict the effects of disturbing the circuit.*

2. 1. 7. *Selverston' s conclusie: CPG' s zijn niet te begrijpen*

Selverston komt tenslotte tot de conclusie dat het met de huidige technieken niet mogelijk zal zijn om de werking van neuronale netwerken volledig te begrijpen. De reden is dat deze technieken niet de mogelijkheid bieden om aan de eisen van de 'minimal list' te voldoen. Hij besluit z'n artikel met een pleidooi voor verdere ontwikkeling en toepassing van technieken die het mogelijk moeten maken om *wel* aan de criteria van de 'minimal list' te voldoen. Deze technieken hebben met elkaar gemeen dat ze de onderzoeker in staat stellen om de aktiviteit van een groot aantal zenuwcellen tegelijkertijd vast te stellen; bovendien moeten ze het mogelijk maken om vast te stellen of een cel deel uitmaakt van een netwerk, zonder dat het nodig is om van die cel de aktiviteit intracellulair te meten [3].

Samenvattend: Selverston komt tot de conclusie dat met gebruikmaking van huidige technieken, CPG' s niet te begrijpen zijn, omdat daartoe een niveau van analyse noodzakelijk is dat niet haalbaar is. Modellen van CPG' s bieden in dit opzicht geen enkele uitkomst. Wellicht bieden nieuwe technische ontwikkelingen perspectieven.
In het onderstaande volgt een samenvatting van het open-peer review: het commentaar van collega' s op het artikel van Selverston.

2. 2. Modellen van neuronale netwerken: verschillende interpretaties

Vrijwel iedereen is gestruikeld over Selverston' s opvattingen over de betekenis van een model van een neuronaal netwerk. Volgens Selverston is het pas zinvol om een model te

---

[3] Eén strategie bestaat eruit om te onderzoeken of het met behulp van genetische technieken (*genetic probes*) mogelijk is om sets van neuronen te identificeren die deel uit maken van één funktioneel netwerk (om zo het probleem op te lossen dat je nooit zeker weet of je wel alle cellen van een netwerk te pakken hebt). Daarnaast is de techniek van zg. *voltage sensitive dyes* in ontwikkeling: hierbij worden kleurstoffen in de cellen gebracht die zodanige eigenschappen hebben, dat ze potentiaal veranderingen in zenuwcellen zichtbaar kunnen maken. Dit zou registratie van aktiviteit van een zeer groot aantal cellen tegelijkertijd mogelijk maken (bv. 100 of meer, in vergelijking tot maximaal 6 met behulp van simultane intracelulaire afleidingen). Tenslotte noemt Selverston de deoxyglucose techniek, een techniek waarbij met behulp van radioaktief gelabeld glucose de metabolische aktiviteit van zenuwcellen gemeten kan worden, waarbij er van uitgegaan wordt dat metabolische aktiviteit correleert met electrische aktiviteit.

ontwerpen van een netwerk wanneer alle gegevens die voortvloeien uit de eis van volledigheid (de *'minimal list'*), bekend zijn. Hiermee ziet Selverston echter over het hoofd dat een model heuristische waarde heeft: het dient als een conceptueel raamwerk om wetenschappelijke vragen te stellen en om een onderzoekstrategie vast te stellen. Bovendien is een model onmisbaar voor interpretatie: zonder model geen betekenisvolle waarnemingen.
Wiens zegt het zo:

> *True, underspecified models are notoriously compliant in supporting preconceived ideas. Still, simulation can explore the potential capabilities and limits of a system in a useful way.*

En Calabrese:

> *Selverston correctly states that poking cells and cataloging properties, though necessary, cannot of themselves lead to mechanistic understanding. What he fails to realize is that the development of testable models is also a necessary step because such models can guide experimentation that will lead to mechanistic understanding.*

Deze auteurs zijn het dus niet met Selverston eens dat je pas moet gaan modelleren wanneer je over alle experimentele gegevens beschikt, integendeel:

> *Network models can, and perhaps should, be formulated even in the absence of any physiological data* (Friesen)

Behalve dat een model van waarde kan zijn omdat het kan aangeven in welke richting een onderzoek voortgezet moet worden (heuristische waarde), heeft het een veel grotere waarde voor het begrijpen van de werking van een netwerk dan Selverston beweert. Larimer en Thompson zeggen hierover:

> *In the absence of some model, the mass of experimental data about neuronal properties and their interconnections will be unintelligible. Experiments performed without a conceptual model can have no intellectual content.*

En Harth:

> *The question is what we mean by 'understanding'. In physics it has become quite clear that the kind of intellectual satisfaction we call 'understanding' almost always involves both simplification and, of necessity, incomplete knowledge. If we would know every cell, every synapse, and the properties of every square micron of neural mebrane, and we would be able to write a computer programme that takes into account all of these data, the result would be no model at all, because it conveys no understanding. It neither simplifies nor unifies.*

Met andere woorden: de voorbeelden die Selverston aanhaalt om duidelijk te maken dat het modelleren van experimentele gegevens verraderlijk kan zijn, vinden de meesten niet alarmerend. Integendeel: het is bij uitstek de manier waarop elk wetenschappelijk onderzoek verloopt.

2. 3. Minimale eisen voor een goed begrip van de werking van een neuronaal netwerk

In het voorgaande werd vooral kritiek geuit op Selverston' s opmerkingen over de beperkingen van modelleren; het volgende hangt daarmee samen, maar spitst zich toe op de *minimal list*. We hebben gezien dat het volgens Selverston noodzakelijk is voor het begrijpen van de werking van een netwerk dat we over alle gegevens beschikken die zijn opgesomd in de *minmial list*. Dit is niet iedereen met hem eens. Sommigen vinden deze criteria te streng, anderen vinden ze niet streng genoeg. Nog weer anderen stellen vast dat een dergelijke lijst van criteria in feite tamelijk willekeurig is.

2. 3. 1. *De minimal list is te streng*

Volgens sommigen is het mogelijk om de werking van een netwerk te begrijpen, zonder over alle gegevens te beschikken, opgesomd door Selverston.
Kaneko merkt bijvoorbeeld op:

> *The question of understanding CPGs is really one of understanding how they produce their output, which does not require an exact knowledge of all components.*

Ook Block ontkent dat alle elementen die genoemd worden in de *minimal list* bekend moeten zijn om een netwerk te kunnen begrijpen. Volgens hem is de enige reden om neurofysiologisch onderzoek aan ongewervelde dieren te doen dat zij als modelsysteem dienen voor hoger ontwikkelde dieren, inclusief de mens. Daarmee bedoelt hij dat je onderzoek doet aan ongewervelde dieren om vervolgens soortgelijk onderzoek te verrichten aan hoger ontwikkelde dieren. In de woorden van Block:

> *The raison d' être for invertebrates in neurobiology is their value as model systems for addressing related problems in the vertebrates.*

Als je de eisen van Selverston zou hanteren, heeft netwerk onderzoek aan hogere dieren geen zin; je kunt immers niet aan die eis voldoen, en dat is noodzakelijk voor het begrijpen van het netwerk. Daaruit volgt dan weer dat onderzoek aan ongewervelde dieren evenmin zin heeft. Het belang van onderzoek aan ongewervelden is immers, aldus Block, afgeleid van de betekenis die het heeft voor soortgelijk onderzoek aan hogere dieren.

Block wenst deze consequentie niet te accepteren, en hij ontkent de noodzaak om aan alle criteria van de *minimal list* te voldoen. Hiervoor in de plaats stelt hij dat het onderzoek aan ongewervelden moet laten zien welke elementen van een netwerk van essentieel belang zijn voor het funktioneren van het netwerk en welke slechts bijkomstig zijn. Het werkelijke doel van dit onderzoek moet zijn dat we op ten duur het funktioneren van een netwerk kunnen afleiden uit bepaalde gegevens, zonder dat *alle* elementen bekend zijn. Het verwerven van dit inzicht is van belang voor het begrijpen van netwerken van hogere diersoorten, waar alle elementen van een netwerk immers onmogelijk geïdentificeerd kunnen worden [4].

2. 3. 2. *De minimal list is niet streng genoeg*

Menig auteur heeft opgemerkt dat de *minimal list* zonder problemen uitgebreid kan worden. Complicerende faktoren, die door Selverston niet genoemd worden, zijn bijvoorbeeld:

- Een zenuwcel wordt steeds beschouwd als een funktionele eenheid; uitlopers van een zenuwcel kunnen echter electrisch geïsoleerd zijn van andere delen van de cel, waardoor het mogelijk is dat processen zich afspelen (bv. afgifte van transmitters) die niet geregistreerd (kunnen) worden in het soma (Grillner).
- Pinsker merkt op dat we niet weten welke hormonen of transmitters allemaal van invloed zijn op het funktioneren van een CPG. Bij experimenten met Ringer oplossingen kunnen componenten afwezig zijn die van groot belang zijn voor de aktiviteit van een netwerk.

2. 3. 3. *De minimal list is willekeurig*

In verband met soortgelijke overwegingen komen sommige auteurs tot de conclusie dat een lijst van criteria waar netwerk-onderzoek minimaal aan moet voldoen tot op zekere hoogte willekeurig is. Székely vat het als volgt samen:

> *There is no guarantee that all items in Selverston' s list represent important parameters; moreover, it is very probable that there are a number of important parameters that we do not appreciate, or can not even envisage, at the present*

---

[4] Cohen heeft hiervoor overigens een oplossing: " The difficulty Selverston raises with regard to studying vertebrates can be circumvented by beginning with the lamprey. The nervous system of the (vertebrate) lamprey qualifies as a 'simple system', while retaining the vertebrate organization. .....Using the lamprey solves none of the problems raised in the invertebrate systems, but it *does* mean that the analysis of a vertebrate CPG can be conducted without adding *additional* problems." Het is natuurlijk de vraag wat nu precies een "simple system" is, en wat "vertebrate organization" is.
Ook Larimer en Thompson besteden aandacht aan de relevantie van onderzoek aan lagere diersoorten voor het begrip van vergelijkbare systemen in hogere diersoorten. Ze concluderen o. a. : 'Escape behaviours in invertebrates and in lower vertebrates are becoming rather well understood. They are, to be sure, less complex than, for example, playing a piano but they are sufficiently complicated to be interesting.'

Lent vraagt zich in dit verband af:

> *What level of knowledge is required for acceptable understanding of the functioning of the CPG? Is this the level of cellular and synaptic mechanisms (as suggested by Selverston), or is it the molecular level? Or do we require a description of the three-dimensional conformation of the membrane bound proteins, molecular linkages between receptor proteins and ion channels in order to obtain a full understanding of the CPG?*

2. 3. 4. *Verklaringen op verschillende niveaus van organisatie*

Er zijn, met andere woorden, verschillende organisatieniveaus waarop we kunnen proberen de werking van een neuronaal netwerk te verklaren. Hiermee stuiten we op de vraag of een verklaring op een bepaald organisatieniveau beter is dan op een ander niveau, en of er een relatie is tussen de verschillende organisatieniveaus.

Volgens Loeb en Marks is het niet zinvol om een systeem op een bepaald niveau van organisatie te analyseren, als de werking van het systeem op het organisatieniveau dat daar boven ligt niet goed wordt begrepen.

> *...there are many equally valid and rigorous levels of explanation. In fact, there may be no limit to the depth to which one might pursue the submolecular bases of a given behaviour. Selverston would seem content to know the membrane properties of the component neurons, but surely the biophysics of the iontophores are part of 'understanding', and beyond that the stereochemistry of membrane proteins beckons.....Yet complete knowledge at the deeper levels is of little help in understanding an emergent property like pattern generation...A thorough understanding of the higher level must inform the investigation into the next deeper layer.*
>
> *The leaping reductionism that tempts us to use any available technique to study a process we can't even properly describe in gross terms leads frequently to those dead ends which Selverston finds so discouraging. In mammalian neurophysiology, the cerebellum is an example of a structure whose basic components and circuitry were made available before there was any real experimental evidence regarding its function, with the consequence that the literature is now full of things that the cerebellum should be doing but is not.*

Loeb en Marks vervolgen:

> *What we are suggesting is that the next step should not be the development of ever-finer anatomical and electrophysiological techniques to study those subcellular processes which, admittedly, do underlie the behaviour. Rather, we must develop better descriptions and manipulations at the systems level and a lengthening list of possible principles of organization. Ironically, this may best be done in vertebrates*

with more complicated and malleable behaviour. Their patterns tend not to be expressed in the all-or-none fashion of invertebrate feeding or escaping but rather to demonstrate experimentally useful ranges of motivation, speed, load compensation, reflex modulation, conditioning, learning etc. Furthermore, the behaviors do not emerge suddenly and completely as invariant instincts, and thus they can be followed through normal versus deprived or altered developmental stages, starting more simply and adapting to fit their environment.

Davis trekt in twijfel of gegevens op een laag organisatieniveau ons begrip kunnen vergroten van het funktioneren op een hoger organisatieniveau:

Even when we have found every single neuron in a given network and mapped all of their synaptic interconnections, it may still be impossible to understand, in any rigorous sense, how the network performs its function.

Als voorbeeld noemt hij:

If we are materialists, then we subscribe to the view that our personalities emerge from nerve cells....And yet it must be self-evident that individual neurons do not possess 'personality' in the same sense in which a human being does. Personality emerges from the interactions of bilions of neurons, in which case it may be analyzable only on the level at which it emerges.

Uit het voorgaande lijkt het duidelijk te worden dat er verschil van mening is over de vraag aan welke eisen moet zijn voldaan, willen we de werking van een neuronaal netwerk kunen begrijpen. Het is aan de neurowetenschappers om op basis van argumenten onderling deze eisen vast te stellen:

What is the nature of the 'proof' that we can adduce to convince ourselves and persuade our colleagues that we have truly attained an unambiguous understanding of a network' s function? or: what is the nature of accepable proof in the neurosciences?

Hoyle concludeert:

There is no absolute standard for understanding, only various levels.

2. 4. Stabiele patronen versus flexibiliteit

Volgens sommige auteurs legt Selverston te veel nadruk op het vermogen van de CPG om zelfstandig een stabiel patroon van aktiviteit te genereren. Een dergelijk concept impliceert dat een CPG een patroon van aktiviteit genereert dat onafhankelijk is van gebeurtenissen in het interne of externe milieu (geen feedback). Voor een volledig begrip

van een CPG moet je echter weten hoe dit patroon veranderd kan worden onder invloed van input van buitenaf.
Ayers merkt bijvoorbeeld op:

> *Investigation of the CPG in the absence of these inputs (from sensory receptors in the peripheral effector organs, or from other CPGs) might give an erroneous view of its capabilities.*

Huber vraagt zich af in hoeverre de motorische aktiviteit die het gevolg is van de aktiviteit van een CPG in een geïsoleerd preparaat identiek is aan het equivalent ervan in het intacte dier. Er is volgens hem geen twijfel aan dat een CPG kan funktioneren in afwezigheid van een input. Dit sluit echter de mogelijkheid niet uit dat we met het bestuderen van een CPG in een geïsoleerd preparaat één of ander artefact bestuderen, en niet een belangrijke neuronale component van een ritmisch gedrag. Er is, aldus Huber, nog geen enkel voorbeeld van een dier dat een motor - output vertoont die relevant is voor z'n gedrag, en die gebaseerd is op uitsluitend de aktiviteit van een CPG. Behalve dat het bestuderen van de effecten van input op de CPG van belang is om te begrijpen hoe de output van een CPG aangepast kan worden aan bepaalde omstandigheden, is dit ook van groot belang voor het begrijpen van de CPG zelf.

Pinsker geeft soortgelijke kritiek:

> *Current neurophysiological approaches to studying CPGs often lack an appropriate ethological frame of reference.*

Onderzoek zou volgens Pinsker moeten beginnen met karakterisering van de ritmische motorische aktiviteit in een dier dat zich vrij kan bewegen, en karakterisering van de patronen van neuronale aktiviteit die hiermee correleren, eveneens in het intacte dier. Deze gegevens moeten als een referentiekader dienen, waarmee gegevens afkomstig van gereduceerde preparaten, voortdurend vergeleken moeten worden.

Met andere woorden: het relevante probleem is niet hoe een geïsoleerd neuronaal netwerk in staat is om een vrijwel onveranderlijk patroon van electrische aktiviteit te genereren, maar: welke eigenschappen hebben neuronale netwerken, die het dier in staat stellen om op flexibele manier te reageren op gebeurtenissen in het milieu? Dit maakt het belangrijk om te zoeken naar flexibiliteit in een netwerk, in plaats van naar mechanismen die verantwoordelijk zouden kunnen zijn voor een onveranderlijke output. Volgens deze kritici is het voor het begrijpen van het funktioneren van een netwerk juist van belang om te onderzoeken waarin de flexibele eigenschappen van een netwerk zijn gelegen.

2. 5. Netwerkfunktie en variabiliteit in het netwerk: redundantie

Zoals we hebben gezien was voor Selverston de belangrijkste vraag, waarvan de beantwoording uiteindelijk moet leiden tot een verklaring van de werking van een CPG: hoe kan een netwerk van neuronen, geïsoleerd van elke input van buitenaf, een gecoördineerd patroon van electrische aktiviteit genereren? Voor Gillette is daarentegen de belangrijkste vraag: hoe is een netwerk van neuronen in staat om een gecoördineerd patroon van electrische aktiviteit te genereren, terwijl er zo' n aanzienlijke mate van variatie optreedt in de structuur van het netwerk als we verschillende dieren van één soort met elkaar vergelijken? Dit element ontbreekt volledig bij Selverston. Volgens Gillette ligt de sleutel tot het begrijpen van de werking van neuronale netwerken juist besloten in het antwoord op de vraag: wat is de oorzaak van deze variatie, en welke eigenschappen stellen netwerken in staat hun essentiële funktie - het genereren van een specifiek patroon van electrische aktiviteit dat verantwoordelijk is voor het produceren van een gecoördineerd gedragspatroon - uit te oefenen ondanks deze variatie in elementen waar de CPG uit opgebouwd is?

2. 5. 1. *Variabiliteit in neuronale netwerken*

Gillette geeft verschillende voorbeelden van dergelijke variaties die waargenomen zijn: een zenuwcel die normaal in enkelvoud aanwezig is in een netwerk, kan in veelvoud voorkomen of juist volledig ontbreken, het vertakkingenpatroon van een zenuwcel kan verschillen, onderlinge verbindingen tussen zenuwcellen kunnen verschillen, membraaneigenschappen van zenuwcellen kunnen verschillen enz. Hoe is het nu mogelijk dat een CPG bestaat, en funktioneert, gezien deze relatief grote mate van variabiliteit? Het antwoord zit, volgens Gillette, in *redundantie*. Dit is het verschijnsel dat een netwerk voor z'n werking, bijvoorbeeld het produceren van een cyclische output, niet afhankelijk is van een enkel mechanisme, maar verschillende mechanismen in zich bergt die gezamelijk werkzaam zijn en elkaar versterken en kunnen aanvullen. Cyclische aktiviteit van een netwerk is bijvoorbeeld voor een deel het gevolg van endogene cyclische aktiviteit van neuronen die deel uitmaken van het netwerk; dit wordt aangevuld door specifieke interakties tussen de neuronen van het netwerk (reciproke inhibitoire inhibities) die produktie van een cyclische aktiviteit ondersteunen en versterken. Zo bezien is het niet de vraag of cyclische aktiviteit toegeschreven moet worden aan individuele zenuwcellen *of* aan netwerken (het onderscheid dat door Selverston wordt benadrukt): het is een eigenschap die op beide manieren is gerealiseerd . In de woorden van Gillette:

> *Cyclic output commonly results from multiple oscillatory properties acting in concert. Such redundancy in oscillatory potential, existing at multiple levels within the network, suggests that the network could tolerate a certain amount of phenotypic variability in the properties and connections of the constituent neurons without seriously affecting the basic features of network output. Indeed, the*

existence of redundancy in oscillatory properties may be an adaptation to accomodate genetic variance in the network elements.

Een soortgelijke suggestie wordt overigens gedaan door Wiens:

Neural circuits operate through redundant mechanisms, none of which may be indispensable, but which all cooperate in the production of an adaptable and failsafe motor rhythm.

In de laatste zin van het citaat van Gillette suggereert hij wat de oorzaak is van de waargenomen variabiliteit: deze is, althans gedeeltelijk, het gevolg van genetische variabiliteit tussen individuen van één soort. Daarnaast kunnen verschillen in individuele ontwikkelingsprocessen verantwoordelijk zijn voor variabiliteit. Gillette stelt dat het optreden van een dergelijke variabiliteit inherent is aan elke vorm van leven, en een voorwaarde is voor evolutie: de aanpassing van levende organismen aan hun milieu als gevolg van selectie. Selectie vooronderstelt variatie. In feite zou het kunnen zijn, dat alle eigenschappen die je nu kunt waarnemen bij levende organismen, eigenschappen zijn die in voldoende mate redundant georganiseerd zijn, dat wil zeggen, de eigenschap hebben om een zekere variabiliteit te kunnen verdragen. Eigenschappen die die mate van variabiliteit niet kunnen verdragen zijn niet stabiel: als ze niet bestand zijn tegen variatie, dan verdwijnen ze weer. Zo bezien, is redundantie een eigenschap die vanzelf ontstaat als gevolg van het mechanisme van variatie en selectie, en die vervolgens zichzelf in stand houdt ('self-sustaining' is).

De hypothese van Gillette is dus: neuronale netwerken vertonen altijd een zekere redundantie; dit is onvermijdelijk, gezien de manier waarop neuronale netwerken tot ontwikkeling zijn gekomen tijdens de evolutie. Alleen als we hiermee rekening houden, kunnen we begrijpen waarom netwerken georganiseerd zijn zoals ze dat zijn. Voor het 'waarom' van een bepaalde organisatie en funktie van een CPG, is het noodzakelijk om de ontwikkeling van de soort (evolutie) en de ontwikkeling van het individuele dier in aanmerking te nemen. Een interessant aspect is dat elementen van de kritiek van Gillette inmiddels nader uitgewerkt zijn in een boek van Gerald Edelman (Neural Darwinism), dat een poging is om de werking van neuronale netwerken te verklaren op een radicaal andere manier dan voordien (Edelman, 1989).

Gillette is overigens niet de enige die deze suggestie doet. Huber maakt ook een opmerking in deze richting, in de vorm van een vraag:

What can we learn about the structural and functional organization of a CPG by studying its development?

Loeb en Marks hierover:

CPGs, particularly in vertebrates, may be inextricably enmeshed in somatosensory perceptual systems whose wiring is specified by accumulated experience operating on self-organizing feature detectors. Our inability to completely specify many of the components and their connections may accurately reflect their nonspecification by nervous system genetics. We then have to consider the possibility that the understanding we really seek is not of the principles of operation of the CPG but of the rules of development and adaptation.

De laatste auteurs leggen dus de nadruk op variabiliteit als gevolg van verschillen in individuele ontwikkeling en het feit dat neuronale netwerken niet volledig gespecificeerd zijn door genetische informatie.

2. 5. 2. *Selverston vs. Gillette: hoe- en waaromvragen*

Wanneer we het stuk van Selverston vergelijken met dat van Gillette, dan blijkt dat Selverston neuronale netwerken wil begrijpen door 'hoe-vragen' te beantwoorden: in het intacte dier kan ik vaststellen dat een onderdeel van een bepaald gedrag bestaat uit contracties van spiergroepen, waarbij die en die spieren in die en die volgorde op die en die wijze contraheren. Die spiercontracties correleren met een bepaald patroon van electrische aktiviteit door motorneuronen; ik kan laten zien dat dat en dat netwerk, met die en die eigenschappen, in staat is om dit patroon van aktiviteit te genereren [5]. Selverston' s onderzoeksstrategie is om eerst alle elementen van het netwerk te identificeren. Elementen van het netwerk zijn gedefinieerd aan de hand van correlatie met de output van het netwerk. De rol van elementen in het netwerk wordt bepaald aan de hand van het effect van verandering van de aktiviteit van het element op de output van het netwerk. Wat dit uiteindelijk oplevert is de kleinste configuratie van zenuwcellen die noodzakelijk èn voldoende is om deze aktiviteit te produceren; een onafhankelijke neuronale eenheid met intrinsieke ordening. Als deze strategie wordt gevolgd dan blijft er echter een vrij groot aantal elementen van het netwerk over, die, voor zover nagegaan kan worden, eigenlijk helemaal niet nodig is voor het funktioneren van het netwerk. Vanuit deze optiek zijn deze elementen redundant: overtollig, want niet strikt noodzakelijk voor het produceren van de netwerk output. De vraag is dan: zijn die elementen echt overtollig? Als verandering van de aktiviteit van een onderdeel van een netwerk (neuron) geen waarneembare

[5] Dit kan aangemerkt worden als een pragmatische interpretatie van wat het begrijpen van de werking van een netwerk inhoudt. Grillner vat dit alsvolgt samen:
The best we will probably ever achieve is a neuronal model that can account for the rhythmicity and the detailed pattern, and that can respond in an appropriate way to the various important inputs that it receives.
Rowell heeft hier zeer uitgesproken ideeën over:
A complete understanding, pragmatically defined, must be the level of understanding adequate to explain all current observations. A deeper understanding than this is a philosophical abstraction. De titel van zijn commentaar heet dan ook: Philosophy leads to pessimism, research to understanding (!)

veranderingen teweeg brengt in de output van het systeem, kan dit o. a. zijn omdat 1) er een zekere redundantie in het systeem is (de aktiviteit van de andere elementen blijft gelijk), of 2) er compensatie optreedt door de overige elementen (de aktiviteit van de andere elementen verandert). Op de laatste mogelijkheid zal ik hier niet verder ingaan; wat betreft de veronderstelde redundantie van netwerkelementen kunnen we in ieder geval drie mogelijkheden onderscheiden:

(1) Die elementen hebben wel degelijk een funktie, alleen wij hebben tot nu toe de funktie over het hoofd gezien. Het produceren van een bepaalde output is niet de enige funktie van het netwerk. Andere mogelijkheden zijn: efferent copies, corollary discharges, coordination, tuning etc. [6]

(2) Als ze echt redundant zijn (dat wil zeggen, ze hebben echt geen enkele funktie) dan betekent dat dat onze impliciete aanname van 'optimal design' niet klopt.

(3) De elementen die als redundant worden aangemerkt hebben wel degelijk een funktie, alleen niet voor het funktioneren van het netwerk in dit dier. De betekenis ervan moet gezocht worden in de ontwikkeling van de soort, in de generatie van voldoende variabiliteit in een systeem waarvan de kernaktiviteit niettemin robuust is (Gillette, Edelman).

Het is van belang om op te merken dat, afhankelijk van welk uitgangspunt hier gekozen wordt, de definitie van een neuronaal netwerk verschilt, het verschijnsel redundantie heel verschillend geïnterpreteerd wordt, en de vraag of eigenschappen toegeschreven kunnen worden aan individuele cellen *of* aan het netwerk in z'n geheel al of niet relevant is [7].

---

[6] Grillner hierover:
It is common to regard the least possible network that can produce a given output as the pattern generator propper, and the remaining parts as redundant. This may be misleading. A cell could subserve a coordinating function in relation to other pattern generators, as well as sending off corollary discharges to relevant parts of the brain.
En Cohen:
The fact that a cell can perturb the rhythm only points to the strength of its connections to important members of the CPG - it does not prove that the cell is itself an element. If the removal of a cell disrupts the pattern, this is strong evidence, but lack of disruption proves nothing. In more complex networks the removal of a cell is likely to have no effect.
Davis merkt hierover op:
In the case of networks we lack the terminology, the concepts, and the criteria to decide what is aceptable proof for a particular neural role or function.
Fentress vraagt zich in dit verband af hoe een netwerk nu precies is gedefinieerd. Welke gevolgen heeft het voor de definitie van een *netwerk*, wanneer 1) een gedrag beïnvloed wordt door verschillende CPG' s? 2) één CPG meerdere typen gedrag reguleert, 3) een zenuwcel deel uitmaakt van meer dan één CPG?

[7] Er zijn verschillende auteurs die het belang van dit laatste onderscheid hebben betwijfeld. Calabrese:
Whether a CPG is 'endogenous' or a 'network', and how this classification relates to the complexity of its output, should not be major concerns..... we must stop thinking in terms of dichotomies between patterns generated as a result of the 'emergent properties' of networks and those generated as a result of the 'endogenous properties' of cells. .... the pattern appears to be the product of both.
Fentress heeft vergelijkbare kritiek: compartmentalization in thought may still exceed the compartmentalization of nature.

Samenvattend luidt de kritiek van Gillette: Selverston begrijpt de organisatie en funktie van een CPG niet, omdat hij volledig over het hoofd ziet dat een CPG moet kunnen werken terwijl er sprake is van een grote mate van variatie tussen individuele dieren van een soort wat betreft de elementen waaruit de CPG is opgebouwd.

2. 6. Geen algemene principes?

Er zijn, tenslotte, een aantal auteurs die sceptisch zijn ten aanzien van de mogelijkheid om enige wetmatigheid te ontdekken in de organisatie van neuronale netwerken, en in de relatie tussen de organisatie van een netwerk enerzijds en de eigenschappen van het gedrag dat door dit netwerk wordt gestuurd anderzijds.

Hoyle schrijft bijvoorbeeld:

> *The manner in which the components are connected and their physiological characteristics were found to be uniquely different for each bit of circuitry examined. The information obtained from a particular bit of nervous tissue was simply not applicable to any other. No principles emerged. Mc Culloch & Pitts (1943) were quite wrong with the only general theory of neural functioning ever published, but after almost half a century nothing is in site that might replace it.*

Naar welke algemene principes van neuronale organisatie zijn we precies op zoek? En welk verband zoeken we tussen kenmerken van gedrag enerzijds, en neuronale organisatie anderzijds? Dumont en Robertson (1986) hebben reden om te veronderstellen dat er wellicht helemaal niet zo' n duidelijk verband is daartussen:

> *Circuits that control apparently similar behaviors may be constructed quite differently.*

Camhi (1984) houdt in dit opzicht nog een slag om de arm:

> *The principles to be sought by neuroethologists take a form such as " under these conditions, animals having these properties are likely to use neurons of this type, organized in this manner". A major goal of neuroethology at present is to uncover as many such principles as possible. Whether these all will be subsumed eventually under some broader unifying principle remains to be seen.* (p. 374).

Voor zover ik kan nagaan lijkt dit inderdaad een goede weergave van de huidige stand van zaken in het netwerkonderzoek.

Samenvattend kunnen we nu stellen dat Selverston zich de vraag had gesteld of de werking van CPG' s te begrijpen is. Uit de commentaren blijkt dat er geen overeenstemming is over de vraag wat nu precies een CPG is, en wat de belangrijkste funktie van een CPG is. Nog belangrijker is het misschien om vast te stellen dat onder

neurofysiologen de meningen uiteenlopen over de vraag wat het begrijpen van de werking van een neuronaal netwerk nu precies inhoudt. Er is verschil van mening over de rol van een model hierbij, en over de gegevens waar we tenminste over moeten beschikken. Ook is er verschil van mening over de vraag wat er nu precies begrepen moet worden. Is de waarneming dat een geïsoleerde CPG een onveranderlijk patroon van electrische aktiviteit kan genereren van belang, en leidt opheldering van de mechanismen die hiervoor verantwoordelijk zijn tot begrip van de werking van een CPG (Selverston)? Of is de waarneming dat een CPG juist in staat is om afhankelijk van de input, een variabele output te leveren van belang, en leidt opheldering van de mechanismen die verantwoordelijk zijn voor deze flexibiliteit juist tot begrip van de werking van de CPG (Huber)? Of is de waarneming dat een CPG in staat is om een vrij constante output te leveren, ondanks aanzienlijke variatie in de opbouw van de CPG van belang, en leidt opheldering van de mechanismen die hiervoor verantwoordelijk zijn tot begrip van de werking van een CPG (Gillette)? En, tenslotte: zijn netwerkeigenschappen überhaupt te begrijpen uit de eigenschappen van de elementen waaruit het netwerk is opgebouwd (Davis)? En zijn er eigenlijk wel algemene principes van neuronale netwerk organisatie (Hoyle)?

Met de weergave van de discussie tussen deze neurowetenschappers heb ik geprobeerd de dynamiek van wetenschapsbeoefening zichtbaar te maken. Het is een discussie over de vraag wat belangrijke, vooralsnog onopgeloste vragen zijn in een tak van de neurofysiologie; het laat zien dat de antwoorden die gegeven worden feilbaar, en altijd voorlopig zijn. In die zin biedt het een goed tegenwicht voor de stelligheid die soms nodig is om een wetenschappelijke uitspraak te poneren. Wetenschappers proberen mee te bepalen welke vragen in hun vakgebied gesteld worden, hoe deze vragen beantwoord worden, en welke verklaringen als het meest adequaat beschouwd worden. Er zijn ongetwijfeld veel faktoren die bepalen of een wetenschapper hierin succesvol is of niet. Dit is een vraagstuk voor de wetenschapssociologie, en valt buiten het bestek van deze inleiding. In het volgende zal ik een toelichting en een samenvatting geven van mijn eigen onderzoek, en ik zal proberen de relatie aan te geven tussen dit onderzoek en de thema' s die in het voorgaande aan de orde waren.

3. HET ADEMHALINGSGEDRAG VAN LYMNAEA STAGNALIS EN DE NEURONALE BASIS

Het onderzoek waarvan in dit proefschrift verslag wordt gedaan, sluit aan bij het soort onderzoek dat besproken wordt door Selverston en zijn collega' s. Het begint met observaties van het gedrag van het dier, en probeert een relatie te leggen tussen het gedrag van het dier en processen die zich afspelen in het zenuwstelsel van het dier. Ook in dit geval betreft het een ongewerveld dier: een zoetwater slak (*Lymnaea stagnalis*, de

poelslak). Het gedrag dat ik bestudeerd heb is het ademhalingsgedrag. Hoewel het dier in het water leeft, heeft het een longholte, en komt het regelmatig naar het wateroppervlak om adem te halen. Het dier doet dat door aan het wateroppervlak de schelp enigszins te kantelen, en vervolgens het ademgat (ook wel: pneumostoom) te openen. Hierdoor kan gasuitwisseling tussen de longholte en de atmosfeer plaats vinden.

3. 1. Bimodale gasuitwisseling

Naast deze gasuitwisseling via de longholte vindt er ook gasuitwisseling plaats over de huid, tussen het bloed en het omringende water. De mate waarin dat laatste gebeurt is afhankelijk van de hoeveelheid zuurstof in het omringende water. Deze kan sterk variëren, in tegenstelling tot de hoeveelheid zuurstof in de lucht, die zeer constant is. We noemen een dergelijke gasuitwisseling bimodaal: gasuitwisseling via de long met de lucht, en via de huid met het omringende water. Wanneer er weinig zuurstof in het water is, is de slak voor z'n zuurstofvoorziening afhankelijk van longademhaling. Hij moet dan in de buurt van het wateroppervlak blijven, om regelmatig adem te kunnen halen.

3. 2. Anaërobe stofwisseling

De slak kan echter ook beperkte perioden overleven zonder zuurstof. Hij kan dit bijvoorbeeld veel beter dan een mens (vele uren, terwijl er bij de mens al na enige minuten zuurstofgebrek onherstelbare schade aan de hersenen ontstaat). In die gevallen schakelt het dier over op een zogenaamde anaëroob metabolisme: een stofwisseling die energie levert zonder zuurstof te verbruiken. Energetisch is dit echter onvoordelig: het levert relatief minder energie dan aërobe stofwisseling. Het stelt de slak echter in staat om perioden van zuurstofgebrek onder water te doorstaan, wanneer hij niet snel genoeg het wateroppervlak weet te bereiken.

3. 3. Fluctuaties in bloed-zuurstofgehalte

Deze bimodale gasuitwisseling impliceert dat beweging van het dier naar en van het wateroppervlak onderdeel is van het ademhalingsgedrag. Deze manier van ademhaling komt bij verschillende diersoorten voor, o. a. kikkers, salamanders en padden, en longvissen. Bij al deze dieren brengt deze manier van ademhalen meestal met zich mee dat de hoeveelheid zuurstof in het bloed van de dieren aanzienlijk fluctueert: tijdens en vlak na ademhaling aan het wateroppervlak is de hoeveelheid zuurstof in het bloed het hoogst. Tijdens het verblijf onder water neemt deze hoeveelheid geleidelijk af. Dieren met een bimodale ademhaling tolereren kennelijk veel beter dergelijke fluctuaties van zuurstofspanning in het bloed dan dieren die alleen in lucht of in water ademhalen. Bij dieren die alleen in water of in lucht ademhalen wordt de zuurstofspanning in het bloed meestal binnen nauwe grenzen zorgvuldig gereguleerd. Hoe sterk de zuurstofspanning in het bloed bij bimodale ademhalers afneemt hangt af van een aantal parameters: het

zuurstofverbruik door het dier, de zuurstofspanning in het omringende water, en de bijdrage van gasuitwisseling over de huid aan de totale zuurstofopname. De laatste is weer afhankelijk van oppervlak en dikte van de huid. Veel dieren die bimodale gasuitwisseling vertonen zijn in staat gebleken om de gasuitwisseling over de huid te reguleren: bij hoge zuurstofspanning in het omringende water is deze gasuitwisseling maximaal, bij lage zuurstofspanning in het water minimaal. Dit laatste is om verlies van zuurstof uit het bloed aan het water te voorkomen. De regulatie gebeurt door de doorbloeding van de huid te verhogen, respectievelijk te verlagen. Het is niet bekend of *Lymnaea* ook in staat is tot een dergelijke differentiële doorbloeding van de huid, afhankelijk van de zuurstofspanning in het water.

In hoofdstuk 3 van dit proefschrift komen een aantal van de hiervoor genoemde aspecten van de ademhaling van *Lymnaea* aan de orde: de relatie tussen longademhaling, zuurstofspanning van het water en het verloop van de zuurstofspanning in het bloed tijdens het verblijf onder water. Bij deze experimenten zijn ook hartslag, anaëroob metabolisme, oriëntatie ten opzichte van het wateroppervlak en duur van ademhaling in relatie tot de duur van de voorafgaande duik bepaald. Bij *Lymnaea* is de zuurstofspanning in het bloed inderdaad tijdens en vlak na longademhaling het hoogst. Deze neemt tijdens het daarop volgende verblijf onder water geleidelijk af. Bij lage zuurstofspanning van het omringende water neemt de zuurstofspanning in het bloed sneller af, en daalt zij naar een lager niveau dan bij normale zuurstofspanning in het omringende water. Het verschil tussen beide kan toegeschreven worden aan gasuitwisseling via de huid. De resultaten suggereren dat er een soort kritische zuurstofspanning in het bloed bestaat: beneden die waarde treedt er anaërobiose op, afname van de hartslag, en zijn de erop volgende perioden van longademhaling aan het wateroppervlak sterk verlengd. Het is mogelijk dat ophoping van zure metabolieten in het bloed (een gevolg van anaëroob metabolisme) een rol speelt bij de totstandkoming van deze effecten. Ook zonder dat de zuurstofspaning in het bloed onder deze waarde daalt, neemt de neiging van het dier om naar het wateroppervlak te bewegen tijdens een duik gaandeweg toe.

3. 4. Faktoren die van invloed zijn op longademhaling

3. 4. 1. *Water- en landdieren*

Wat betreft de faktoren die ademhaling in bimodale gasuitwisselaars reguleren is niet zo erg veel bekend. In de literatuur wordt doorgaans een scherp onderscheid gemaakt tussen dieren die in lucht, en dieren die in water ademhalen. De eerste categorie leeft in een omgeving waarin een constante, en hoge zuurstofspanning heerst; de ademhaling wordt normaal vooral beheerst door koolzuur, dat afkomstig is van de stofwisseling en dat voor een groot deel het zuur-base evenwicht van het bloed beheerst. Receptoren die gevoelig zijn voor veranderingen in gasspanningen meten vooral veranderingen in de bloedbaan

(en in de hersenen), en niet in het milieu: daar treden eigenlijk geen veranderingen op in zuurstof- of koolzuurspanning. Waterdieren daarentegen, raken via de huid het koolzuur heel makkelijk kwijt aan het water. Deze dieren leven echter in een omgeving waarin het zuurstofaanbod relatief laag, en bovendien variabel is. In een bepaald volume water dat met zuurstof verzadigd is zit veel minder zuurstof dan in een zelfde volume atmosferische lucht. Bovendien kan onder bepaalde omstandigheden plaatselijk in het water gedurende bepaalde tijd de zuurstof voor een groot deel verdwijnen. Bij dieren die in water ademen wordt de ademhaling dan ook vooral beheerst door zuurstof. Van een aantal dieren is bekend dat de zuurstofspanning niet alleen in het bloed, maar ook in het milieu (water) wordt gemeten.

3. 4. 2. *Lymnaea: tussen water en land*

Gezien dit onderscheid tussen water- en landdieren, is het de vraag hoe de situatie is bij dieren die in beide media ademhalen. Hier zijn niet zo veel gegevens over, en dit is een van de aspecten die ik voor *Lymnaea* nader heb onderzocht. Het blijkt dat *Lymnaea* in dit opzicht zowel kenmerken van water- als van landdieren heeft: er zijn aanwijzingen dat *Lymnaea* de zuurstofspanning in het water kan meten (een kenmerk van waterdieren), maar er zijn ook aanwijzingen voor effecten van koolzuurgas op de ademhaling van *Lymnaea* (een kenmerk van landdieren). Deze aspecten komen aan de orde in hoofdstuk 2 van dit proefschrift. Bij lage zuurstofspanning van het omringende water blijkt het dier sterker geneigd om naar het wateroppervlak te bewegen dan bij normale zuurstofspanning van het omringende water. De perioden dat het dier aaneengesloten onder water verblijft zijn daarmee korter en de frequentie waarmee het dier aan het wateroppervlak komt om longademhaling uit te voeren hoger. Aan het wateroppervlak blijkt bij lage zuurstofspanning van de ingeademde lucht de neiging van het dier om weer terug te keren onder water geringer dan bij normale zuurstofspanning. Bovendien is dan de neiging om het ademgat te openen en weer te sluiten verhoogd. Dit heeft tot gevolg dat het dier aan het wateroppervlak veel, kortdurende ademhalingsbewegingen uitvoert.

De bewegingen van het ademgat kunnen ook door tactiele- en schaduwstimuli worden beïnvloed: 1) het doorbreken van het wateroppervlak is een noodzakelijke voorwaarde voor het openen van het ademgat; wanneer het ademgat onder water komt sluit het altijd; 2) bij lichte aanraking van het dier sluit het ademgat, als onderdeel van een algemene terugtrekreaktie van het dier; 3) bij sterkere aanraking of bij schaduwstimuli kan het dier zijn ademgat openen en de lucht in de achterliggende longholte eruit persen. Daardoor kan het dier zich volledig terugtrekken in z'n schelp, en zinkt het naar de bodem van het water. Dit is een vluchtreaktie van het dier, die het ook vertoont wanneer het aangevallen wordt door natuurlijke predatoren.

Bewegingen van het ademgat zijn dus betrokken bij een drietal verschillende gedragingen: ademhaling, terugtrekgedrag en vluchtgedrag. Deze bewegingen worden veroorzaakt door verschillende typen van stimulatie.

3. 5. Ademhaling: interferentie met andere typen van gedrag

Een ander aspect van bimodale ademhaling is, dat deze potentieel nogal interfereert met andere gedragingen. *Lymnaea* brengt het grootste deel van z'n leven onder water door. Onder water legt het dier eieren (die op het land zouden uitdrogen) en vindt het dier voedsel. *Lymnaea* gaat alleen van tijd tot tijd naar het wateroppervlak om adem te halen. U moet zich voorstellen dat voor iedere keer dat u adem wilt halen, u even de deur uitmoet om een luchtje te scheppen. Het went op den duur waarschijnlijk wel, maar het is toch steeds weer een lastige onderbreking van de dingen die u binnen aan het doen bent. Britse onderzoekers die dit verschijnsel bij een watersalamander hebben onderzocht, gaven hun artikel de titel 'To breathe or not to breathe' mee. In de hoofdstukken 4 en 5 worden deze aspecten beschreven voor *Lymnaea*. Uit de resultaten van de experimenten blijkt dat tijdens eileg of tijdens eten, *Lymnaea* relatief lang onder water kan blijven. *Lymnaea* lijkt als het ware de signalen, die het dier normaal gesproken naar het wateroppervlak doen bewegen om adem te halen, te negeren.

3. 6. Het *hoe* en *waarom* van het ademhalingsgedrag van *Lymnaea*: evolutietheorie en neuronaal netwerkanalyse

Het gedrag van *Lymnaea*, zoals ik dat hierboven heb beschreven, kan tal van vragen oproepen. Zoals eerder aangegeven, is het zinvol om onderscheid te maken tussen hoe- en waarom vragen: *hoe* doet *Lymnaea* dit? Welke eigenschappen van het dier stellen het in staat om dit gedrag op deze manier te vertonen en *waarom* gedraagt het zich zo en niet anders?

3. 6. 1. *Waarom zo?*

Om met het laatste te beginnen: zoals al eerder opgemerkt, wordt voor de beantwoording van deze vraag een beroep gedaan op de evolutietheorie. Via het proces van variatie en selectie ontstaan er allerlei levensvormen die op een bepaalde manier gebruik maken van de mogelijkheden die geboden worden door het natuurlijke milieu. Al die verschillende levensvormen gaan deel uitmaken van het milieu, en beïnvloeden als zodanig op hun beurt weer het selectieproces. Camhi (1984) heeft dit idee verwoord met betrekking tot de manier waarop bij dieren eigenschappen zijn ontstaan waardoor dieren nuttig gebruik kunnen maken van informatie in hun omgeving:

> *It is safe to say that if there is useful information contained in some form of environmental energy, some animal has probably evolved a way to detect and utilize*

it. Evolution, then, has been highly opportunistic in exploiting environmental energies to meet behavioral needs. (p. 373)

Nu wordt over het algemeen aangenomen dat de zoetwater slak *Lymnaea* in de evolutie ontstaan is uit landslakken (McMahon, 1983). De overgang van een landleven naar een leven in het zoete water zal gepaard zijn gegaan met de nodige aanpassingen, bv. aanpassing van water- en zouthuishouding. Net zoals de omgekeerde beweging, evolutie van water naar land, gepaard gaat met omvangrijke aanpassingen. Bij deze overgang van land- naar waterleven heeft *Lymnaea* longademhaling, typisch voor landdieren, behouden. De hypothese is nu, dat dit *Lymnaea* in staat heeft gesteld om te leven in zoetwater met lage zuurstofspanning. Dit is een milieu waarin betrekkelijk weinig andere dieren kunnen overleven, met als gevolg dat bv. de predatiedruk laag is: de kans dat *Lymnaea* opgegeten wordt door een ander dier is klein. In dit water leeft *Lymnaea* vooral van planten en plante-resten. Op deze manier zouden de processen van natuurlijke variatie en selectie aanleiding hebben gegeven tot de ontwikkeling van de soort *Lymnaea*, die op zodanige manier aangepast is aan een specifieke natuurlijke omgeving, dat deze tot op de dag van vandaag als soort heeft kunnen overleven.

Uit mijn eigen onderzoek blijkt onder andere dat de koolzuurspanning van de omgeving van invloed is op het ademhalingsgedrag van *Lymnaea*. Deze gevoeligheid voor koolzuurspanning wordt beschouwd als een typische eigenschap van landdieren. Dit zou opgevat kunnen worden als een bevestiging van het vermoeden dat *Lymnaea* zich heeft ontwikkeld van een land- tot een zoetwaterslak, waarbij niet alle aanpassingen aan het landleven verloren zijn gegaan.

3. 6. 2. *Hoe is Lymnaea in staat dit gedrag te vertonen?*

Zoals al opgemerkt, is de werkhypothese van neurofysiologisch onderzoek dat elk aspect van het gedrag een weerspiegeling is van fysisch-chemische processen in het zenuwstelsel. Het ademhalingsgedrag van *Lymnaea*, zoals boven beschreven, is al vrij complex, en slechts enkele aspecten ervan konden nader uitgezocht worden op het niveau van zenuwcellen; netwerkanalyse is een moeizame en tijdrovende geschiedenis.

In hoofdstuk 6 worden de resultaten beschreven van experimenten, waarbij in het zenuwstelsel gezocht is naar zenuwcellen die een specifieke respons vertonen op verandering van zuurstofspanning in het longgebied. Dergelijke cellen zijn inderdaad gevonden. Ze zijn kennelijk betrokken bij de overdracht in het zenuwstelsel van informatie over de zuurstofspanning in het longgebied. Een opmerkelijk gegeven is, dat onder de zintuigcellen die in *Lymnaea* verantwoordelijk zijn voor de oriëntatie in het zwaartekrachtsveld (de statocystcellen) er een aantal is waarvan de aktiviteit beïnvloed wordt door verandering van zuurstofspanning in het longgebied. Dit zou een voorbeeld

kunnen zijn van de beïnvloeding van de aktiviteit van een zintuig (in dit geval het evenwichstzintuig) door een ander zintuig (zuurstof receptoren). Dit verschijnsel noemen we efferente innervatie. Het bestaan van dit mechanisme in *Lymnaea* werd al eerder geopperd door Janse (1981). De betekenis van dit mechanisme is dat het voor een deel verantwoordelijk zou kunnen zijn voor het feit dat oriëntatie in *Lymnaea* afhankelijk is van de zuurstofspanning in de omgeving: onder zuurstof-arme condities is *Lymnaea* geneigd om naar het wateroppervlak te kruipen, terwijl het onder zuurstof-rijke condities geneigd is om naar de bodem van het water te kruipen.

In hoofdstuk 7 wordt een beschrijving gegeven van zenuwcellen waarvan de aktiviteit correleert met bewegingen van het pneumostoom. Sommige van deze cellen veroorzaken bij electrische stimulatie gecoördineerde bewegingen van het pneumostoom. Onder deze cellen bevinden zich cellen die verhoogde aktiviteit vertonen bij lage zuurstofspanning in het longgebied. De eigenschappen van deze cellen wijzen op een rol in de regulatie van ademhalingsbewegingen van het pneumostoom. Andere cellen zijn specifiek gevoelig voor aanraking van het huidgebied rond het pneumostoom, of voor een schaduw-stimulus, en vertonen een alles-of-niets respons. De eigenschappen van deze cellen wijzen op een rol in de regulatie van pneumostoombewegingen tijdens terugtrek- of vluchtgedrag. De contacten tussen deze verschillende cellen zijn, voor zover aanwezig, betrekkelijk zwak, zodat het niet erg voor de hand ligt om van één netwerk van zenuwcellen te spreken.

In dit deel van het onderzoek bleek dat twee zenuwcellen -in de vakliteratuur VD1 en RPD2 genoemd- mogelijk een centrale rol spelen bij de regulatie van pneumostoom-bewegingen. Het zijn relatief grote zenuwcellen, met een wijd patroon van vertakkingen. De cellen zijn peptiderg, dat wil zeggen dat de stof die door deze cellen geproduceerd en afgegeven wordt, en waarmee de aktiviteit van andere cellen binnen of buiten het zenuwstelsel beïnvloed wordt, een eiwit is (een peptide). De electrische aktiviteit van deze cellen neemt sterk af wanneer de zuurstofspanning in het longgebied laag is. Onder deze omstandigheden neemt de ademhalingsaktiviteit (de frequentie van pneumostoom-openings- en sluitbewegingen) toe. Wanneer de electrische aktiviteit van VD1 en RPD2 experimenteel -d. w. z. met behulp van electrische stroom- verlaagd wordt, kan dit effect nagebootst worden: de ademhalingsaktiviteit neemt dan toe. De hypothese is, dat VD1 en RPD2 andere cellen stimuleren, die een remmend effect hebben op de ademhalingsaktiviteit. Als VD1 en RPD2 niet aktief zijn (bv. als gevolg van lage zuurstofspanning), dan valt deze remming weg, en ontstaat de mogelijkheid voor ademhalingsaktiviteit.

In hoofdstuk 8 wordt tenslotte getoond welke transmitters in belangrijke mate de aktiviteit bepalen van de zenuwcellen die betrokken zijn bij de regulatie van ademhalingsbewegingen.

3. 7. Relatie met overig onderzoek binnen de Vakgroep Organismale Dierkunde

Binnen de Vakgroep Organismale Dierkunde waar ik mijn promotieonderzoek heb verricht, zijn verschillende aspecten van bovengenoemde cellen, VD1 en RPD2 onderzocht. Daardoor is de aard van de peptiden die door deze cellen geproduceerd en afgegeven worden bekend (Bogerd, 1992). Uit dit onderzoek is gebleken dat deze peptiden overeenkomst vertonen met de peptiden van een zenuwcel in een verwant diersoort, namelijk R15 in de zeeslak *Aplysia*. Van deze cel is bekend dat deze betrokken is bij regulatie van ademhaling en water- en zouthuishouding. Uit het onderzoek van Bogerd is ook gebleken dat een peptide dat door VD1 en RPD2 geproduceerd wordt, een stimulerend effect heeft op de hartslag van *Lymnaea*. Daarnaast is in detail het vertakkingenpatroon van beide cellen uitgezocht, zowel binnen als buiten het zenuwstelsel (Kerkhoven, 1991). Uit dit onderzoek is gebleken dat de cellen vertakkingen hebben in het longgebied, in de nabijheid van het pneumostoom. Ook zijn er vertakkingen gevonden in de nabijheid van het hart. Zoals in alle dieren, bestaan er ook in *Lymnaea* nauwe relaties tussen vegetatieve funkties als ademhaling, hartslag en water-en zouthuishouding. De onderzoeksresultaten van Kerkhoven en Bogerd versterken de hypothese dat VD1 en RPD2 betrokken zijn bij regulatie van vegetatieve funkties in *Lymnaea*.

Daarnaast zijn veranderingen die optreden in electrische eigenschappen van VD1 en RPD2 in relatie tot leeftijd bestudeerd door Wildering (1992). Dit laatste onderzoek heeft plaats gevonden in het kader van het onderzoek naar veroudering. Door de gegevens uit dit onderzoek in verbinding te brengen met gegevens over de funktie van deze cellen, hopen we op ten duur inzicht te krijgen in de wijze waarop veranderingen in electrische eigenschappen van deze cellen die optreden in relatie tot leeftijdstoename doorwerken in funktieveranderingen.

Tenslotte wordt op dit moment onderzoek vericht aan beide cellen, waarbij electrische aktiviteit wordt afgeleid in levende, vrij-bewegende dieren (zg. in-vivo registraties; Smelik en Ter Maat, persoonlijke mededeling). Dit stelt de onderzoekers in staat om vast te stellen of gedrag van het dier, vastgelegd op videoband, in verband gebracht kan worden met electrische aktiviteit van VD1 en RPD2. Als zodanig kan het hypotheses toetsen, die voortvloeien uit onderzoek aan semi-intacte preparaten.

3. 8. Nogmaals: Neurowetenschap in aktie

Veel van de problemen en vragen die aan de orde komen in het stuk van Selverston en in het commentaar daarop, doen zich ook voor met betrekking tot mijn eigen onderzoek. Met

het onderzoek zijn een aantal zenuwcellen geïdentificeerd die deel uitmaken van een netwerk van cellen die pneumostoombewegingen reguleren. Het is moeilijk vast te stellen of hiermee het netwerk redelijk beschreven is, of dat er nog belangrijke elementen ontbreken. Wel kan ik vaststellen dat met mijn gegevens nog lang niet is voldaan aan de eisen van de *minimal list* van Selverston. Niettemin heb ik de resultaten voorlopig weergegeven in een model van een netwerk, waarvan overigens niet getoetst is of het inderdaad de experimentele gegevens kan nabootsen. In het model wordt het bestaan van reciproke contacten tussen een aantal cellen verondersteld, zonder dat elk van die cellen geïdentificeerd is; het bestaan van sommige cellen wordt afgeleid uit het optreden van karakteristieke synaptische input op volgcellen. Dit is in ieder geval een illustratie van het feit dat een model inderdaad heuristische waarde kan hebben: het biedt de mogelijkheid om gericht te onderzoeken of de veronderstelde contacten er zijn of niet.
Zoals we hebben gezien betwijfelt Huber of het zinvol is om de electrische aktiviteit van geïsoleerde netwerken te bestuderen. Tijdens mijn eigen onderzoek bleek ook dat netwerken van zenuwcellen andere patronen van electrische aktiviteit kunnen genereren in geïsoleerde zenuwstelsels dan in zenuwstelsels waar perifere structuren intact zijn gelaten. In dat opzicht lijkt mij de kritiek van Huber en Pinsker terecht. De relevantie van metingen aan neuronale aktiviteit in geïsoleerde zenuwstelsels voor het gedrag van het intacte dier is niet vanzelfsprekend.

Pinsker heeft ook opgemerkt dat in veel gevallen gegevens over het gedrag van het intacte dier ontbreken, waar de gegevens van de neuronale netwerkanalyse aan gerelateerd zouden moeten worden. Met deze kritiek ben ik het van harte eens. Ook tijdens mijn eigen onderzoek bleek de neiging tot netwerkanalyse groot, terwijl er in feite nog onvoldoende bekend was van het gedrag. Dit maakte het in mijn ogen noodzakelijk om aanvullende gedragsobservaties te verrichten. Ook dit heeft echter beperkingen. De gedragsobservaties werden in het laboratorium verricht; de relevantie van deze waarnemingen voor het gedrag van het dier in z'n natuurlijke leefmilieu spreekt niet vanzelf. Er is, gegeven de grote hoeveelheid kennis op het gebied van het fiunktioneren van het zenuwstelsel van *Lymnaea*, relatief weinig bekend over de ecofysiologie van het dier.

Gillette benadrukt dat het opvallend is dat hetzelfde netwerk in verschillende preparaten steeds hetzelfde patroon van electrische aktiviteit kan genereren, ondanks aanzienlijke variatie tussen die netwerken. De variaties waarvan hij voorbeelden geeft, zijn wij vrijwel zeker tegen gekomen tijdens het onderzoek, hoewel we dat niet systematisch hebben onderzocht. Vaak lijken cellen in preparaten te ontbreken, of zijn er juist meer van, en vertakkingenpatronen van cellen kunnen sterk variëren van preparaat tot preparaat. Het uitgangspunt bij dit type onderzoek is echter, dat verschillende preparaten onderling identiek zijn. Dat zal, mede dankzij een zeer goed gecontroleerde kweek van proefdieren, waarschijnlijk in grote lijnen ook het geval zijn. Uitzonderingen worden als anomalieën beschouwd, waar geen betekenis aan toe geschreven moet

worden. In het licht van de theorie van Gillette krijgen deze waarnemingen wel degelijk betekenis. Een aardig voorbeeld van wat men 'theorie-gebonden waarneming' noemt.

Gillette benadrukte ook het belang van redundantie: het verschijnsel dat eigenschappen van een netwerk van zenuwcellen op verschillende, elkaar aanvullende manieren gerealiseerd zijn. Tijdens mijn onderzoek bleken er veel zenuwcellen te bestaan die patronen van electrische aktiviteit vertonen die gecorreleerd zijn met bewegingen van het pneumostoom. Verandering van aktiviteit (kunstmatig onderdrukken van aktiviteit of stimuleren van aktiviteit) had echter geen merkbare invloed op de pneumostoom–bewegingen. De funktie van deze cellen is niet bekend. Zijn deze cellen, of hebben ze subtielere funkties die mij zijn ontgaan? Ik houd het voor mogelijk dat beantwoording van deze vraag ons dichter brengt bij een begrip van wat neuronale netwerken doen, hoe ze dat doen, en waarom ze dat zo doen, en niet anders.

Tenslotte kan ik, met Hoyle, vaststellen dat er vooralsnog eigenlijk geen duidelijke, algemene principes van neuronale organisatie zijn, die een verband leggen met kenmerken van het gedrag dat ze reguleren.

GECITEERDE LITERATUUR:

Bogerd J. Generation of neuropeptide diversity by alternative splicing in two giant neurons, VD1 and RPD2, of the mollusc *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.

Camhi JM. Neuroethology. Nerve cells and the natural behavior of animals. Sinauer Associates Publishers, Massachusetts, (1984).

Dumont J en Robertson RM .Neuronal circuits: an evolutionary perspective. Science **233**: 849 - 853 (1986)

Edelman GE. Neural Darwinism. The theory of neuronal group selection. Oxford University Press, 1989.

Janse C. The effect of oxygen on gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **142** A (1981): 51 - 59.

Kerkhoven RM, RP Croll, J v Minnen, J Bogerd, MD Ramkema, H Lodder en HH Boer. Axonal mapping of the giant peptidergic neurons VD1 and RPD2 located in the central nervous system of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain research **556** (1991): 8 - 16.

Latour B. Wetenschap in aktie. Bert Bakker, 1988. Vertaling van Science in action, 1987.

Mc Mahon RF. Physiological ecology of freshwater pulmonates. In: The Mollusca, Vol. 6: Ecology. Academic Press, 1983.

Selverston AI. Are Central-Pattern Generators understandable? The behavioural and Brain Sciences **3** (1980): 535 - 571.

Wildering WC. Synaptic plasticity in the central nervous system of adult *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.

CHAPTER 2

MULTIMODAL SENSORY CONTROL OF RESPIRATORY BEHAVIOUR IN THE PULMONATE FRESHWATER SNAIL *LYMNAEA STAGNALIS* (MOLLUSCA, GASTROPODA)

SUMMARY

The freshwater pulmonate snail *Lymnaea stagnalis* shows an intermittent mode of breathing: Periods of aerial ventilation at the water surface are alternated by periods of submergence. During the ventilatory period, one or more opening movements of the pneumostome occur at the water surface. This mode of ventilation implies that orientation of the animal with respect to the water surface is an important component of the behaviour.

During hypoxia, the intervals between successive bouts of aerial ventilation were decreased and the animals carried out many, relatively short lasting ventilatory movements during each bout. The effect of hypercapnia on ventilatory behaviour depended on the ambient PO_2: at normoxia, hypercapnia resulted in a decrease of the intervals between successive bouts of aerial ventilation, and the animals carried out few, but relatively long lasting respiratory movements during each bout. At hypoxia, however, hypercapnia resulted in an increase of the number of respiratory movements during each bout; the duration of intervals between two successive bouts of aerial ventilation and the duration of respiratory movements were not different from those, observed during normocapnic hypoxia.

When the PO_2 of the ambient water and the inspired air were varied independently, low PO_2 of the ambient water resulted in a decrease of the interval between two successive bouts of aerial ventilation. In contrast, low PO_2 of the inspired air resulted in an increase of the number of respiratory movements during each bout, and did not affect the interval between successive respiratory bouts.

It is concluded that hypoxia and hypercapnia affect ventilatory behaviour in *Lymnaea* in specific ways, distinct from each other. These effects are probably mediated by separate pathways that interact with each other. O_2-chemosensitivity probably resides both internally as well as externally. Apart from the effects on pneumostome movements that result from the factors, mentioned above, pneumostome movements are also affected by tactile and shadow stimuli. These stimuli induce withdrawal or escape; in both of these behaviours, pneumostome movements are involved.

INTRODUCTION

The physical properties of water and air impose quite different demands on ventilatory mechanisms and their associated neural control (Piiper, 1982; Dejours, 1981, pp. 23 - 35, 185 - 220). Air breathing animals live in a relatively stable environment as regards partial pressures of O_2 and CO_2 (PO_2 and PCO_2, respectively). Chemoreceptive structures, sensitive to changes in PO_2 and PCO_2 which mediate adjustments of respiration and circulation, are located internally.

Water breathing animals live in a much more unstable environment with respect to PO_2 and PCO_2. Therefore, in these animals internal changes of PO_2 and PCO_2 may also result from fluctuations of these parameters in the ambient water. Consequently, in order to show appropriate adaptations, water breathing animals should be able to distinguish between PO_2 and PCO_2 changes originating internally or externally (Johansen, 1970). Generally, respiration and circulation in water breathing animals are more profoundly affected by changes in PO_2 than by changes in PCO_2 (Dejours, 1981). Taken together with the necessity to distinguish between internal and external changes, this observation suggests that in water breathing animals external and internal O_2 chemoreceptive structures are present. Indeed, both internal- and external O_2 chemosensitivity have been demonstrated in aquatic crustaceans and fish (Crabtree and Page, 1973; Massabuau and Burtin, 1984; Taylor, 1982; Daxboeck and Holeton, 1978; Shelton, Jones and Milsom, 1986; Milsom, 1991).

Apart from these species that are either exclusively air- or water breathing animals (unimodal gas exchange), a number of animal species is capable of gas exchange in both, water and air. This bimodal gas exchange occurs in a wide variety of animals (fish, amphibia, reptiles, annelids, crustaceans and molluscs, *cf.* Dejours, 1981, pp. 87 - 89). With respect to the factors that control respiration, bimodal gas exchangers may resemble either air- or water breathing animals. This depends on whether the animals are primarily terrestrial or aquatic (Shelton and Boutilier, 1982; Truchot, 1990). A common feature among bimodal breathers is that surfacing and submergence of the animal are a necessary component of ventilatory behaviour. This implies that orientation of the animal with respect to the water surface plays a role in air breathing behaviour.

The fresh water pulmonate snail, *Lymnaea stagnalis* is such a bimodal breather (Jones, 1961). In the present study, we investigated the factors that control air breathing behaviour in this species. We studied effects of environmental changes on the two components of air breathing behaviour: orientation of the animal with respect to the water surface and ventilatory movements, carried out at the water surface. The environmental changes consisted of changes in PO_2 (characteristically affecting respiration of water-breathing animals), PCO_2 (characteristically affecting respiration in air-breathing animals), or both. We varied the PO_2 of the water and the inspired air independently, and

determined the effects of these treatments on haemolymph PO_2. This was used as a means to distinguish between effects, mediated by internally and externally located chemoreceptive structures. In our experiments, the PCO_2 of the ambient water and the inspired air could not be varied independently, because of the rapid exchange of CO_2 between the two media. In the first part of the paper we describe the organization of air breathing behaviour in *Lymnaea* and the effects of tactile stimulation on ventilatory movements. The second part of the paper deals with the effects of changes in environmental PO_2 and PCO_2 on air breathing behaviour.

MATERIALS AND METHODS

Experimental animals and conditions

Adult specimens of *Lymnaea stagnalis* (L.) , shell length ca. 30 mm were used. They were bred under laboratory conditions at a 12 - 12 hr light - dark regimen (van der Steen, van den Hoven and Jager, 1969). During the experiments, snails were observed in jars containing 500 ml of tap water (20° C). The height of the water level was 6 cm. Before placing animals in the jars, the water was equilibrated with gas. After transferring the snails, the jars were closed with a glass plate, leaving sufficient space for aerial ventilation. This space was gently flushed (gas flow 250 ml/min) with the appropriate gas mixture (inspired air), without disturbing the snails. Prior to observations, the snails were acclimatized for a period of 15 min. Gas composition was controlled with mass flow controllers (Brooks Instruments, model 5850 TR), connected to a Four Channel Control Unit (Brooks Instruments, model 5878). Pure O_2, pure N_2 and calibrated gas mixtures of 5 % CO_2 in O_2 or in N_2 (Hoekloos) were used. At the start and the end of the observation period PO_2 and PCO_2 of the water were measured. O_2 rich water always had a PO_2 between 130 - 150 Torr, and O_2 poor water between 25 - 45 Torr.

Snails were subjected to the following conditions: 1) O_2 rich water, free access to atmospheric air (normocapnic normoxia); 2) 5 % CO_2 in water and inspired air, normoxic PO_2 in water and air (normoxic hypercapnia); 3) O_2 poor water, low PO_2 of inspired air (25 Torr) (normocapnic hypoxia); 4) 5 % CO_2 in water and inspired air, hypoxic PO_2 in water and air (hypercapnic hypoxia); 5) O_2 poor water, free access to atmospheric air; 6) O_2 rich water, low PO_2 of the inspired air (25 Torr). Because of the high solubility of CO_2 in water, no attempt was made to study the effects of changes in PCO_2 of either the water or the inspired air seperately. Five different snails were subjected separately to each condition.

Behavioural observations and measurements

The effects of the aforementioned conditions on the respiratory behaviour were studied by observing freely moving snails. During a period of one hour migratory and ventilatory

behaviour was monitored, and a number of behavioural parameters were measured (see Fig. 3, schematically representing these parameters). When snails were at the water surface, a bout of pneumostome opening movements could occur. The number (N_p) and duration (T_{vent}) of such individual movements were measured. Summation of T_{vent} gives the ventilatory period (T_{vp}) during a bout. Intervals between bouts of ventilatory activity (T_{nvp}) were determined by measuring the time lapse between the end of the last pneumostome movement of a bout and the beginning of the first opening movement of the next bout. Overall effects of possible changes in these parameters of ventilatory behaviour were measured by determining the time spent to gas exchange (% ventilation) as the fraction of the total observation time.

At the end of the observation period (75 min. after the start of the incubation), haemolymph PO_2 and heart rate were measured. Heart rate was measured by visual inspection of the heart through the transparant shell. PO_2 of haemolymph and water were measured with a polarographic O_2 electrode (Instech laboratories, model 125/05) connected to a Single Channel Oxygen Amplifier (Instech laboratories, 102 B/230). To check for changes in the response time of the electrode and for drift the output signal of the amplifier was continuously fed to a calibrated recorder. Haemolymph PO_2 was measured immediately after collection. During the measurement of the PO_2, the haemolymph was in contact with the atmosphere. Hence, some O_2 diffusion may have taken place during the measurement between the haemolymph sample and the atmosphere, resulting in measured values that are slightly higher than the actual values. Our values are, however, compatible to values of haemolymph PO_2 of other snails, reported in the literature, suggesting that diffusion of O_2 did not affect the measurement in a substantial way (Brix *et al.*, 1979; Ainslie, 1980; De Fur and Lukowiak, 1982). Haemolymph was collected by forcing the snails to retract into the shell through tactile stimulation of the foot. The animals then expel haemolymph from the hemal pore (Bekius, 1972). The amount of haemolymph, thus collected, varies between 0.4 and 0.7 ml. This represents at least 50 - 75 % of the total volume of haemolymph (cf. Van Aardt, 1968).

PCO_2 of the water was measured with a micro carbon-dioxide electrode (Microelectrodes, model number MI 720), connected to a pH meter (Knick, type 646). The following solutions were used for calibration: tap water equilibrated with pressurized air (20 % O_2), sodium dithionite solution (10^{-2} M $Na_2S_2O_4$ in tap water), (0 % O_2), and tap water, equilibrated with pure N_2 (0 % CO_2) or with N_2 with 5 % CO_2 (5 % CO_2).

In a number of experiments, responses to tactile and shadow stimuli were studied. Tactile stimuli were delivered with a hand driven glass probe (tip diameter 1 mm).

Statistical analyses

The data were statistically analyzed, using a two-way analysis of variance. Firstly, we determined the statisitical significance of the effects on the different parameters of 1) the decreased PO_2 of the ambient water and the inspired air, 2) the increased PCO_2 in both media, and 3) the interaction between the two factors. Secondly, we determined the statistical significance of the effects of the decrease of the PO_2 in the ambient water and the inspired air separately.

RESULTS

Air breathing behaviour

Air breathing behaviour in *Lymnaea stagnalis* consists of opening- and closure movements of the pneumostome, carried out at the water surface. This behaviour is preceded by movement of the animal towards the water surface and followed by the return towards the lower depths of the water.

Migration towards the water surface ends when the tentacles touch the water surface. The animal then shows a slight withdrawal reaction and stops locomoting. At this moment, respiratory movements start. The shell is pulled anteriorly and turned at the same time in such a way, that the pneumostome area, located at the right side of the body, is exposed to the atmosphere (Figs. 1 A and B). Movements of the pneumostome result in the formation of a kind of siphon as long as the pneumostome is under water. Opening of the pneumostome occurs when it is exposed to the atmosphere (Fig. 1 C; start of a bout). When the shell is lowered into the water, and the pneumostome becomes submerged again, the pneumostome closes immediately. When the animal moves along the water surface, it may also open its pneumostome (Fig. 1 D). The animal terminates aerial ventilation by returning to the lower depths of the water again (Fig. 1 E; end of the bout). In addition, the animals are capable of air breathing while suspended from the water surface (Fig. 1 F). In both types of lung ventilation the extent to which the pneumostome opens, varies from slight opening to complete, wide opening.

Effects of tactile stimulation on pneumostome opening and closure

We tested whether the stimuli, experienced by the animal during contact of the pneumostome area with the water surface could be mimicked by local tactile stimulation using a glass probe (tip diameter 1 mm) of the pneumostome area or adjacent mantle area. Tactile stimulation at low intensities, however, always induces withdrawal of the animal, accompanied by closing contractions of the pneumostome. When the pneumostome is open, tactile stimulation results in immediate closure of the pneumostome. When the pneumostome is closed, it results in flattening of the outer fold of the pneumostome against the mantle edge.

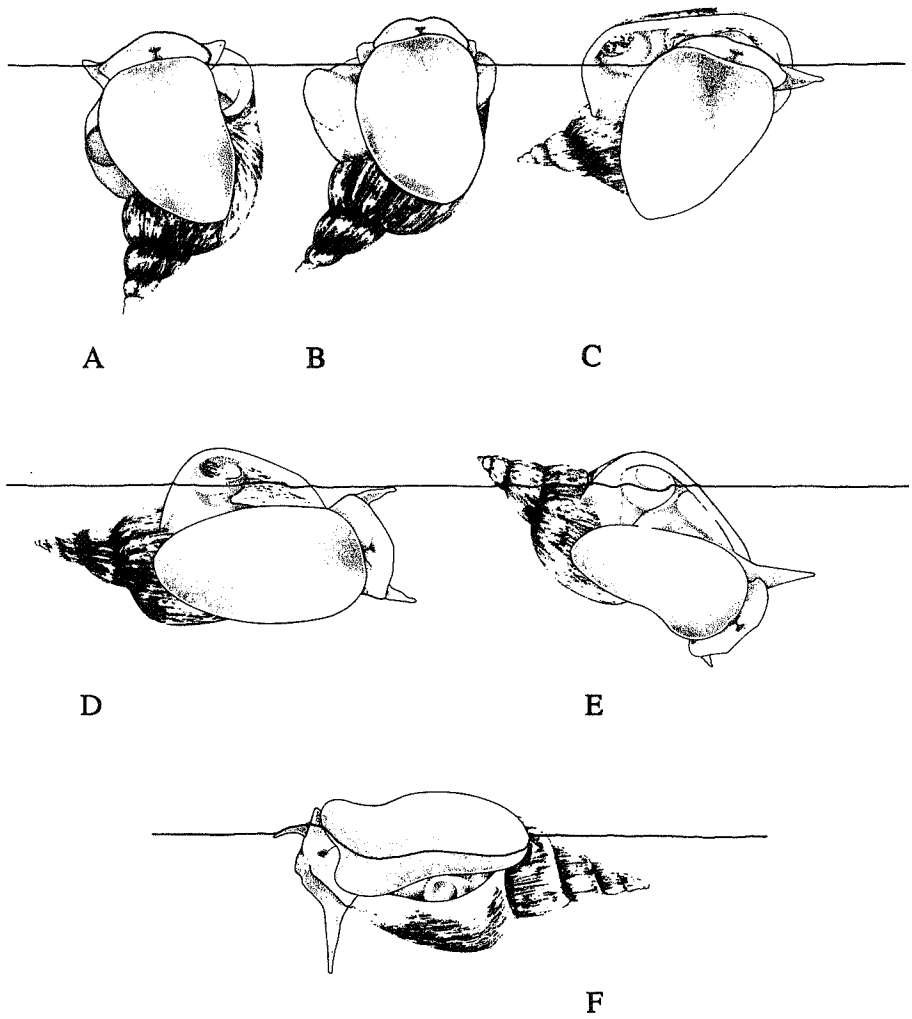


Figure 1. Sequence of events observed during aerial ventilation in *Lymnaea* while the animal is attached to a substrate at the water surface (A - E), and aerial ventilation during suspension of the animal from the water surface (F). For details see text.

Stronger tactile stimuli result in complete retraction of the animal into the shell. During this movement, the animal expels the contents of the lung cavity. When, at the time of stimulus application, the pneumostome is closed, expulsion of air is accompanied by rapid wide opening and subsequent rapid closure of the pneumostome. This wide, sudden opening movement of the pneumostome differs clearly from the opening movement observed during ventilation, which is more slowly and gradually. When the pneumostome is already open during stimulation, expulsion of air is followed

immediately by closure of the pneumostome. Subsequent stimulation within a period of up to *ca.* 2 min fails to elicit the rapid opening response again. Apparently this is an extremely fast habituating response. During the unresponsive period, strong aswell as low-intensity stimulation elicits closing contractions of the pneumostome. The rapid type of pneumostome movements can also be evoked by a shadow stimulus. These rapid opening- and closure movements of the pneumostome constitute the first part of escape behaviour, during which the animal expels the contents of the lung cavity and sinks to the bottom of the water (Bekius, 1972).

These observations show that local tactile stimuli can not induce the pneumostome movements, observed during respiration. They can, however, induce a special type of pneumostome opening- and closing movements which are part of escape behaviour.

As it appeared to be impossible to induce ventilatory movements by local tactile stimulation and surfacing appeared to be an important trigger for pneumostome movements, we tried to mimick tactile stimulation involved in surfacing by varying the water level. It appeared that this 'mimicked surfacing' is a powerful stimulus for pneumostome opening. In 61 out of 74 cases (observations on 5 different snails) 'surfacing' resulted in pneumostome opening. The stimulus became even more powerful (100 %) when prior to the stimulus snails had been unable to breathe for a period of 15 min by placing a glass plate on top of the jar, containing the snail, or when they were forced to breathe air with a low PO_2 prior to submergence. Apparently, a factor that is contingent on breathing behaviour sets the sensitivity of the opening response (see below). The reversed stimulus, increasing the water level, mimicking submergence, always resulted in closing of the open pneumostome. It is concluded that the water surface provides a stimulus to the animal which could not be mimicked by gentle mechanical stimulation using a hand-driven probe, as far as the slow opening response - occurring during ventilatory movements- is concerned. On the other hand, closing movements are less demanding as regards the type of mechanical stimulation. In addition to this, there exists a special pneumostome response which can be elicited by strong tactile stimulation as well as shadow stimuli, and which habituates extremely fast. This movement consists of a fast wide opening, followed immediately by closing.

Effects of environmental hypoxia and normoxic and hypoxic hypercapnia

In the following, we studied the effects of a decrease of water and inspired air PO_2 (hypoxia) and / or increase of water and air PCO_2 (hypercapnia) on breathing behaviour, haemolymph PO_2 and heart rate. Subsequently, the effects of low PO_2 of the ambient water and the inspired air were studied. Haemolymph PO_2 was measured to ascertain whether changes in external conditions influencing breathing behaviour could be mediated by stimulation of internal- or external receptors. Heart rate was measured to obtain information on the respiratory condition of the animal.

Changes in breathing behaviour in response to environmental changes could occur in several ways. The interval between respiratory bouts (non-ventilatory period, T_{nvp}), the total duration of ventilation during a bout (ventilatory period, T_{vp}), the number of pneumostome openings per bout (N_p), or the duration of individual pneumostome openings (T_{vent}) could vary. Changes in one or more of these parameters could result in an overall change of the total time spent to ventilation (% ventilation).

Table 1. Mean values and standard deviations of parameters of respiratory behaviour, heart rate and haemolymph PO_2 in *Lymnaea* during normocapnic normoxia (I), hypercapnic normoxia (II), normocapnic hypoxia (III) and hypercapnic hypoxia (IV). Means and standard deviations of observations on 5 different snails. The last three columns represent estimated p - values of the main effects of hypoxia and of hypercapnia and of interactions between the two factors. The mean values in columns I - IV correspond with traces I - IV in Figure 2.

PARAMETERS	TREATMENT				ESTIMATED P-VALUES		
	NORMAL P_{O_2}		LOW P_{O_2}				
	normal P_{CO_2}	high P_{CO_2}	normal P_{CO_2}	high P_{CO_2}	main effect P_{O_2}	main effect P_{CO_2}	inter-action
% ventil.	6.3 ±1.3	28.3 ±1.8	44.6 ±7.9	36.3 ±56.4	<0.001	<0.001	<0.001
T_{nvp} (sec)	535.1 ±69.9	126.2 ±40.0	142.6 ±55.3	150.6 ±46.9	<0.001	0.003	0.006
T_{vp} (sec)	36.7 ±7.6	100.7 ±7.4	195.1 ±51.8	421.8 ±200.7	<0.001	<0.001	>0.05
Np (number/bout)	1.29 ±0.31	1.32 ±0.18	11.34 ±3.79	17.64 ±4.10	<0.001	0.02	0.04
T_{vent} (sec)	24.4 ±6.1	78.5 ±13.5	17.8 ±2.1	23.4 ±6.7	<0.001	<0.001	<0.001
Heart rate (beats/min)	34.6 ±2.1	33.0 ±2.4	20.0 ±1.6	19.8 ±1.5	<0.001	>0.05	>0.05
P_{O_2} haemol. (Torr)	52.0 ±3.2	51.7 ±1.8	20.7 ±1.9	20.6 ±1.5	<0.001	>0.05	>0.05
	I	II	III	IV			

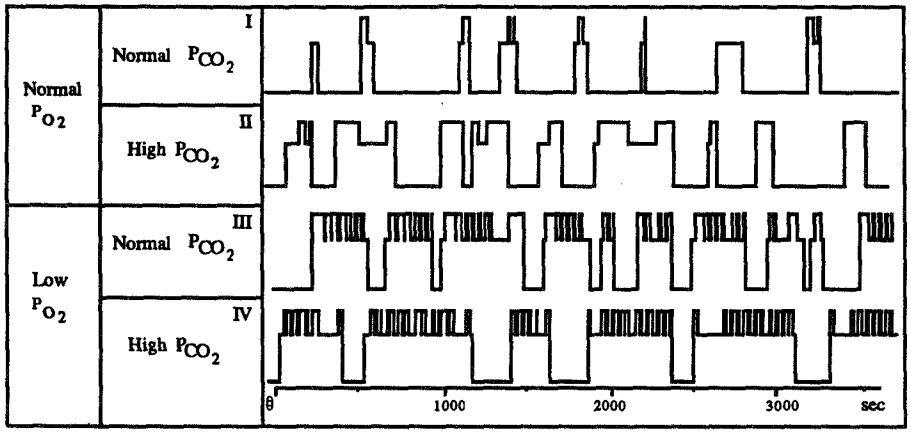


Figure 2. Schematic representation of migratory behaviour (surfacing and submergence) and ventilatory behaviour (opening- and closure movements of the pneumostome) at the water surface of *Lymnaea* at normoxia (I), hypercapnia (II) hypoxia (III), and hypoxic hypercapnia (IV). Each trace is derived from observations on a single animal, representative for the particular group. Hypoxia and hypercapnia both tend to increase the percentage of time the animals spent ventilating through a decrease of the intervals between successive ventilatory bouts (decrease of the non-ventilatory period) and an increase of the duration of ventilation during each bout (increase of the ventilatory period). However, the increase of the ventilatory period is brought about in entirely different ways: At hypoxia, the *number* of respiratory movements during a bout is increased, whereas during hypercapnia the *duration* of individual respiratory movements is increased. When hypoxia and hypercapnia are combined, the ventilatory period is increased as compared to hypoxia through a further increase of the number of respiratory movements during a bout.

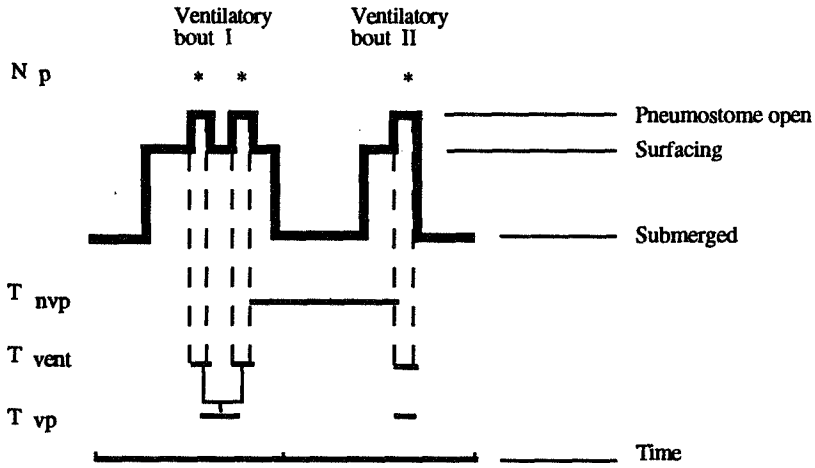


Figure 3. Explanation of parameters which were used for the description of ventilatory behaviour. Two bouts of aerial ventilation are shown, separated by a non-ventilatory interval (T_{nvp}). During the first bout, two ventilatory opening movements of the pneumostome occur (asterisks, $N_p = 2$), each of particular duration (T_{vent}); the duration of the ventilatory period during the bout (T_{vp}) is the sum of the durations of each T_{vent}. During the second bout of aerial ventilation, only one ventilatory pneumostome opening occurs (asterisk, $N_p = 1$). In this case, the duration of the ventilatory movement (T_{vent}) equals the duration of the ventilatory period during this bout (T_{vp}).

For the sake of convenience, the data of the experiments where the effects of hypoxia and hypercapnia were studied are presented in Table 1 and Fig. 2, the data of the experiments where the effects of the PO_2 of the ambient water and the inspired air were studied separately are presented in Table 2 and Fig. 4. Both Tables present the mean values and standard deviations of the measurements and the results of the two-way ANOVA. Note that due to this representation of the data, the data in columns I and III of Table 1 are the same as those in columns V and VIII, respectively, of Table 2. The Figures are traces schematically representing migratory behaviour (surfacing and submergence) and respiratory behaviour (pneumostome movements) of the animals during the different conditions. Each trace is derived from observations on a single animal, representative for the particular group.

Effects of hypoxia and hypercapnia on migratory and ventilatory behaviour in Lymnaea

The effects of hypoxia and hypercapnia on the time lapse between two successive bouts of ventilation (T_{nvp}) were clearly dependent on each other. From the data in Table 1 it can be seen that hypercapnia resulted in a considerable decrease of the T_{nvp} at normoxia. At hypoxia, T_{nvp} was already decreased as compared to normoxia, and hypercapnia did not result in a further decrease of this parameter.

The effects of hypoxia and hypercapnia on the number (N_p) and duration (T_{vent}) of individual pneumostome openings were also dependent on each other. In Table 1 it can be seen that hypoxia resulted in a considerable increase of the number of pneumostome openings during a bout; this effect was stronger during hypercapnia than during normocapnia. Hypercapnia resulted in a considerable increase of the duration of individual pneumostome openings during normoxia; this effect was virtually absent during hypoxia. Together, these changes resulted in a significant increase of the duration of the ventilatory period during each bout (T_{vp}). This is true of hypoxia as well as hypercapnia.

The effects of hypoxia and hypercapnia on the percentage of the total observation time the animals spent ventilating (% ventilation) were also interdependent; hypoxia resulted in an increase of this parameter, but the effect was much stronger at normocapnia than at hypercapnia.

A comparison of the traces in Figure 2 representing migratory and ventilatory behaviour of *Lymnaea* during hypercapnia (II) and during hypoxia (III) with the traces of these behaviours during normoxia (I) illustrates the effects, mentioned above (the mean values in columns I - IV in Table 1 correspond with the schematic representations I - IV of migratory and ventilatory behaviour in Figure 2; in Figure 3 the relation between these traces and the parameters in Table 1). At hypoxia as well as hypercapnia the intervals between successive ventilatory bouts were decreased. At the surface, however, ventilatory behaviour in the two conditions was quite different: during hypercapnia (trace

II) the animals carried out few, but relatively long-lasting ventilatory movements during each bout . In contrast, during hypoxia (trace III) the animals carried out many, but relatively short-lasting ventilatory movements during each bout. When hypoxia and hypercapnia were combined (trace IV), a further increase of the durations of ventilation during each bout occurred.

In addition to these effects on behavioural parameters, hypoxia, but not hypercapnia, resulted in a decrease of heart rate and haemolymph PO_2 of the animals as compared to those of the animals during normocapnic normoxia.

Effects of the PO_2 of the ambient water and the inspired air on migratory and ventilatory behaviour in Lymnaea

Table 2 shows that the PO_2 of the ambient water significantly decreased the intervals between successive ventilatory bouts (non-ventilatory period, T_{nvp}). It did not affect significantly any of the parameters of ventilatory behaviour at the surface, nor did affect heart rate or haemolymph PO_2. The effect of the PO_2 of the ambient water on the percentage ventilation depended on the PO_2 of the inspired air: at low PO_2 of the inspired air this effect was more conspicuous than at normal PO_2 of the inspired air.

In contrast, the PO_2 of the inspired air had a significant effect on all of the parameters of ventilatory behaviour at the surface: total durations of ventilation during a bout (ventilatory period, T_{vp}), and number and duration of individual ventilatory movements of the pneumostome (N_p and T_{vent}, respectively). It also significantly affected heart rate and haemolymph PO_2. The PO_2 of the inspired air did not significantly affect the intervals between successive ventilatory bouts. Finally, it affected significantly the fraction of the total observation time the animals spent ventilating (% ventilation). As already mentioned, with respect to the latter parameter significant interactions occurred between the PO_2 of the ambient water and the PO_2 of the inspired air.

The differences between the effects of decreased PO_2 of the ambient water and decreased PO_2 of the inspired air on ventilatory behaviour can also be seen in Figure 4 (the mean values in columns V - VIII in Table 2 correspond with the schematic representations V - VIII of migratory and ventilatory behaviour in Figure 4). Comparing the traces VI and V in Figure 4, shows that during low PO_2 of the ambient water, the intervals between successive ventilatory bouts were slightly decreased (non-ventilatory period, T_{nvp}); there was no significant difference in ventilatory behaviour at the surface in the two conditions. In this condition, heart rate and haemolymph PO_2 were not affected.

Comparing the traces VII and V shows that during low PO_2 of the inspired air many relatively short lasting ventilatory movements of the pneumostome were carried out at the water surface (increase of the number and decrease of the duration of individual ventilatory movements of the pneumostome, N_p and T_{vent}, respectively). In contrast, the

intervals between successive ventilatory bouts were not affected (T_{nvp}). In this condition, heart rate and haemolymph PO_2 were decreased.

If low PO_2 of the ambient water and the inspired air are combined, a combination of the foregoing effects occurred: decrease of the intervals between successive ventilatory bouts (T_{nvp}), increase of the durations of ventilation during each bout (T_{vp}), increase of the number (N_p) and decrease of the duration (T_{vent}) of individual ventilatory pneumostome movements.

Table 2. Mean values and standard deviations of parameters of respiratory behaviour, heart rate and haemolymph PO_2 in *Lymnaea* during normal PO_2 of the inspired air and ambient water (V), normal PO_2 of the inspired air, *low* PO_2 of the ambient water (VI), *low* PO_2 of the inspired air, normal PO_2 of the ambient water (VII) and *low* PO_2 of the inspired air and ambient water (VIII). Means and standard deviations of observations on 5 different snails. The meann values in columns V - VIII correspond with traces V - VIII in Figure 4. The last three columns represent estimated p - values of the main effects of the PO_2 of the ambient water and of the PO_2 of the inspired air and interactions between the two factors.

PARAMETERS	TREATMENT				ESTIMATED P-VALUES		
	Normal P_{O_2} air		low P_{O_2} air				
	normal P_{O_2} water	high P_{O_2} water	normal P_{O_2} water	high P_{O_2} water	main effect P_{O_2} air	main effect P_{O_2}	inter-action
% ventil.	6.3 ±1.3	13.3 ±2.9	23.1 ±8.4	44.6 ±7.9	<0.001	<0.001	<0.01
T_{nvp} (sec)	535.1 ±69.9	283.8 ±25.5	486.4 ±188.9	142.6 ±55.3	<0.001	>0.05	>0.05
T_{vp} (sec)	36.7 ±7.6	51.8 ±17.0	175.7 ±84.5	195.1 ±51.8	>0.05	<0.001	>0.05
Np (number/bout)	1.29 ±0.31	1.60 ±0.41	9.80 ±4.50	11.34 ±3.79	>0.05	<0.001	>0.05
T_{vent} (sec)	24.4 ±6.1	33.1 ±11.2	18.3 ±4.2	17.8 ±2.1	>0.05	<0.001	>0.05
Heart rate (beats/min)	34.6 ±2.1	34.4 ±2.5	21.4 ±2.7	20.0 ±1.6	>0.05	<0.001	>0.05
P_{O_2} haemol. (Torr)	52.0 ±3.2	52.3 ±3.4	20.8 ±1.9	20.7 ±1.9	>0.05	<0.001	>0.05
	V	VI	IVII	VIII			

In summary: low PO_2 of the ambient water and low PO_2 of the inspired air both affect ventilatory behaviour in *Lymnaea*, but in different ways: low PO_2 of the ambient water affects migratory behaviour, low PO_2 of the inspired air affects ventilatory behaviour at the water surface. These effects are to the major part independent of each other. Only at low PO_2 of the inspired air, heart rate and haemolymph PO_2 are decreased.

In addition to the changes described above, a decreased PO_2 in the inspired air resulted in additional, qualitative changes in repiratory behaviour. In this condition, snails often moved slightly out of the water and regularly carried out lung ventilatory movements at or above the water surface. Usually, the following characteristic sequence of behavioural events was observed. First, the shell was pulled forward (Figs. 5 A and B). Then, while keeping the shell in this position, the pneumostome opened widely (Fig 5 C). Next, the shell moved downwards again to its original position, while the pneumostome remained open (Fig. 5 D). This sequence of events was terminated by closure of the pneumostome and could be repeated several times. Similar changes occurred during normocapnic and hypercapnic hypoxia. In addition, locomotory activity of the snails gradually decreased during hypercapnic hypoxia. The animals, however, never became completely quiescent.

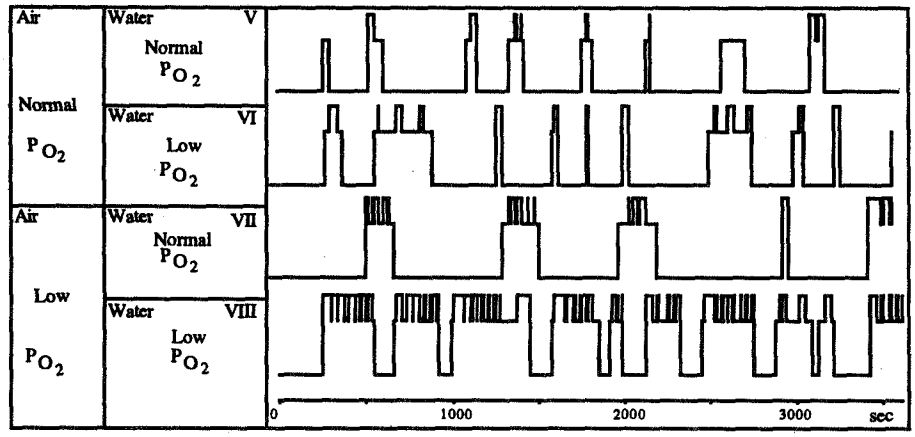


Figure 4. Schematic representation of migratory behaviour and ventilatory behaviour at the water surface of *Lymnaea* at normoxia (V), low PO_2 of the ambient water (VI), low PO_2 of the inspired air (VII) and low PO_2 of ambient water *and* inspired air (VIII). Each trace is derived from observations on a single animal, representative for the particular group. Low PO_2 of the ambient water resulted in an increase of the percentage of time the animals spent ventilating through a decrease of the intervals between successive ventilatory bouts (decrease of the non-ventilatory period); in this condition, the animals maintained normoxic values of haemolymph and heart rate. In contrast, low PO_2 of the inspired air resulted in an increase of the percentage of time the animals spent ventilating through an increase of the ventilatory period, which, in turn, resulted from a strong increase of the number of respiratory movements during a bout. In this condition, haemolymph PO_2 and heart rate were significantly decreased. At low PO_2 of water and air a combination of these effects occurred.

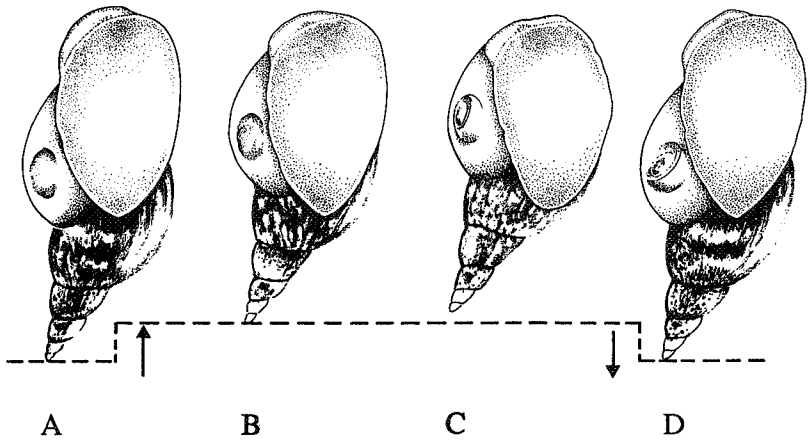


Figure 5. Sequence of events observed during aerial ventilation in *Lymnaea* while the animal is attached to a substrate at the water surface during low environmental PO_2. For details see text.

The characteristic sequence of pneumostome- and shell movements indicates that shell movements play a role in gas exchange between the lung cavity and the atmosphere. The anterior movement of the shell possibly results in an increase of the atmospheric pressure in the lung cavity. Thus, the release of the contents of the lung cavity to the atmosphere during the subsequent pneumostome opening will be facilitated. When the shell returns to its original position, it possibly generates a negative pressure gradient between the lung cavity and the atmosphere. As the pneumostome is kept open during this movement, this will facilitate inspiration. Changes in atmospheric pressure in the lung cavity, accompanying respiratory movements have also been described for *Helix* (Dale, 1974).

DISCUSSION

The results of the present study show that ventilatory behaviour in *Lymnaea* consists of orientation and migration towards the water surface, opening movements of the pneumostome and accompanying shell movements at the surface and return of the animal to the lower depths of the water again. Changes in ambient PO_2 and changes in ambient PCO_2 affect the time-budgetting of the animals over underwater activities and aerial ventilation at the surface. Changes in ambient PO_2 could result in changes in heart rate and haemolymph PO_2; changes in ambient PCO_2 did not affect these parameters. In the description of the behaviour we have distinguished between three possible states: i. the animal is submerged, ii. the animal is at the water surface with pneumostome closed, iii. the animal is at the water surface with pneumostome open (Figure 6). Whether the animal will open its pneumostome at the surface, the number and the duration of these openings

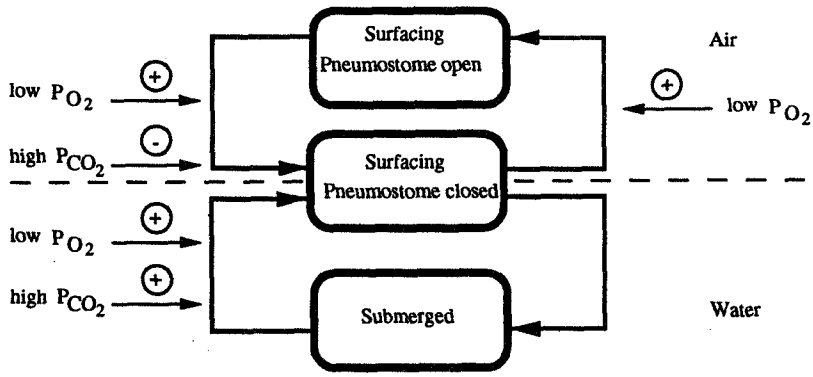


Figure 6. Model of aerial ventilatory behaviour of *Lymnaea*, distinguishing three different states and ambient PO_2 and PCO_2 affecting the transition rates between these states.

can be considered as reflecting the tendencies of the animal to open and close its pneumostome. Several factors will affect these tendencies. Our data suggest that low PO_2 of the inspired air increases the tendency of the animal to open, as well as to close its pneumostome, resulting in an increase of the number, and a decrease of the duration of pneumostome opening movements. At normoxia, high PCO_2 resulted in an increase of the duration of pneumostome opening movements, but not the number. This suggests that this condition primarily decreases the tendency of the animal to close the pneumostome, but does not affect the tendency to open it. In a similar fashion, the occurrences and durations of periods of submergence can be considered to reflect the tendency of the animal to start and terminate a dive. Since low PO_2 and high PCO_2 of the water both resulted in a decrease of the intervals between successive bouts of ventilation, these factors probably increase the tendency of the animals to orientate and move towards the water surface.

What do these observations mean for the control of ventilatory behaviour in *Lymnaea*? Jones (1961) has shown that after a period of submergence, and immediately before a pneumostome opening occurs, the PO_2 in the lung cavity is low and the PCO_2 is normal as compared to atmospheric values; immediately after termination of a ventilatory bout, PO_2 and PCO_2 in the lung cavity are nearly atmospheric. Our data are consistent with the following model: at the surface, a low PO_2 in the lung cavity (or the adjacent circulatory system) results in a relatively high tendency to open the pneumostome; if gas exchange results in restoration of atmospheric PO_2 in the lung cavity - that is, if the PO_2 of the

inspired air is atmospheric and gas exchange occurs effectively - the tendency to carry out an additonial ventilatory movement by opening its pneumostome decreases, and the tendency to terminate the ventilatory bout by returning to the lower depths of the water will increase. If, on the other hand, the PO_2 in the lung cavity or adjacent circulatory system is not restored to atmospheric levels (due to ineffective diffusion or due to low PO_2 of the inspired air) the tendency to open the pneumostome remains high and the tendency to terminate the ventilatory bout remains low. High ambient PCO_2 decreases the tendency to close the pneumostome and to terminate a ventilatory bout. In this model, ambient PO_2 and PCO_2 are considered as probabilistic factors, affecting the transition rates between the different states of the behaviour, rather than deterministic factors, which could not explain for the observations that, for instance, also at low ambient PO_2 and high PCO_2, the animals *do* terminate bouts of aerial ventilation and start subsequent dives.

Although Jones' s data (1961) indicate that *Lymnaea* loses its metabolically derived CO_2 mainly *via* the skin, our data indicate that ventilatory behaviour in *Lymnaea* is still under the control of PCO_2. This type of control would allow for a function of aerial ventilation to eliminate metabolically derived CO_2; *Lymnaea* may have retained this feature as it evolved from a terrestrial ancestor to the primary aquatic animal which it is now (see below).

Nature and localization of chemoreception

High PCO_2 in the ambient water and the inspired air will result in correspondingly elevated PCO_2 in the haemolymph (De With and Van der Wilt, unpublished observations). Consequently, it is not possible to infer from the present data the localization (circulatory system, lung cavity, skin) of chemosensititvity mediating these effects. The effects of low PO_2 of the inspired air on ventilatory movements may be mediated by O_2 chemosensitivity, located in the lung cavity or in the circulatory system (since haemolymph PO_2 was also decreased in this condition). In addition, it is conceivable that internal pH changes may play a role in the onset of the responses to low PO_2 of the inspired air and to hypercapnia, as both conditions are known to result in decreased haemolymph pH (Burton, 1983; De With, unpublished results). A decreased internal pH alone cannot, however, account for the responses during hypoxia and hypercapnia, since the two responses are distinctly different from each other.

Though by no means conclusive, the effects of decreased PO_2 of the ambient water are consistent with a mediation by externally located O_2-chemosensitivity. The presence of such O_2 chemosensitivity in the skin of *Lymnaea* has already been postulated by Janse (1981). If correct, it would imply that as compared to normal ambient PO_2, at low PO_2 of the ambient water the tendency of *Lymnaea* to orientate and move towards the water surface is always increased. The significance of this may be that it ensures that the animal

will in general remain in closer proximity to the water surface; this is important since in this condition the animal relies mainly or even exclusively on aerial ventilation.

Taken together, our data are consistent with the presence of chemosensitivity at different sites, affecting different aspects of ventilatory behaviour. The effects of hypoxia and hypercapnia partly interact with each other. This is reminiscent of the situation in vertebrates, where similar interactions occur when hypoxic and hypercapnic stimuli are combined; here, indications have been obtained for the occurrence of multiplicative interactions at the level of the carotid chemoreceptor (O' Regan and Majcherczyk, 1982; Boutilier, 1988).

Mechanosensorily mediated effects on pneumostome movements; involvement of pneumostome in ventilatory- and escape behaviour

Apart from changes in ambient PO_2 and PCO_2 that affect ventilatory behaviour, the behaviour is also controled by factors that are likely to be mediated by mechanosensory elements. Our data show that when *Lymnaea* is moving towards the water surface, in most instances surfacing stops the vertical migration and starts ventilatory activity consisting of pneumostome and shell movements. Probably mechanical stimulation encountered during surfacing plays a role in the triggering of these movements. Ventilatory activity cannot be evoked by tactile stimulation using a hand driven probe, while tactile stimulation by manipulation of the water level appears to be an appropriate trigger for ventilatory movements. In contrast, hand driven probe tactile stimulation and shadow stimuli can evoke pneumostome movements of the type that normally occur during escape behaviour. Pneumostome movements, associated with escape behaviour differ clearly from ventilatory movements: they are quick and consist of complete opening and closing while ventilatory movements are slow and gradual. Apparently the pneumostome serves as an effector organ in different types of behaviour which differ in the stimulations that can elicit the characteristic movements.

A somewhat similar situation exists in the marine gastropod, *Aplysia*. In this species, a wide range of stimuli of different modality is capable of eliciting contractions of the gill. Among these stimuli are mechanical stimulation of the siphon or mantle shelf (Kupfermann and Kandel, 1969), changes in illumination (Eberly, Kanz, Taylor and Pinsker,1981) and changes in ambient PO_2 and PCO_2 (Koester, Dieringer and Mandelbaum, 1979; Croll, 1985; Levy, Achituv and Susswein, 1989). Depending on the type of stimulus, a primarily defensive or respiratory role has been attributed to these gill contractions in *Aplysia*.

Resemblance to control of ventilation in unimodal air- and water breathing animals

With respect to the effects of increased PCO_2 on respiratory behaviour, *Lymnaea* resembles air breathing animals, where changes in PCO_2 may profoundly affect

respiratory activity. With respect to the putative presence of both external- and internal O_2 chemosensitive elements, however, *Lymnaea* resembles water breathing animals. This may be related to the evolutionary development of freshwater pulmonate snails. They have probably reevolved an aquatic mode of life from a terrestrial ancestor. Among them primitive, nearly terrestrial species are found, as well as advanced, purely aquatic species (Mc Mahon, 1983). *Lymnaea* represents an intermediate between the two extremes, being largely aquatic, but having retained a pulmonary mode of gas exchange.

REFERENCES

van Aardt, W. J. (1968). Quantitative aspects of the water balance in *Lymnaea stagnalis*. Neth. J. Zool. **18**: 253 - 312.

Ainslie R. C. (1980). The quantitative role of haemocyanine in the response of abalone (genus *Haliotis*). J. Exp. Zool. **211**: 87 - 99.

Bekius, R. (1972). The circulatory system of *Lymnaea stagnalis* (L.). Neth. J. of Zool. **22** (1): 1 - 58.

Boutilier, R. G. (1988). Control of breathing in bimodal breathers: Amphibia. Can. J. Zool. **66**: 6 - 19.

Brix O., G. Lykkeboe and K. Johansen (1979). Reversed Bohr- and Root shifts in haemocyanin of the marine prosobranch, *Buccinum undatum*: adaptations to a periodically hypoxic habitat. J. Comp. Physiol. **B129**: 97 - 103.

Burton, R. F. (1983). Ionic regulation and water balance. In: A. S. M. Saleuddin and K. M. Wilbur (Eds.), The Mollusca, Vol. 5, Physiology, part 2. Academic Press, New York.

Crabtree, R. L. and Page, C. H.(1974). Oxygen - sensitive elements in the book gills of *Limulus polyphemus*. J. Exp. Biol. **60**: 631 - 639.

Croll, R. P. (1985). Sensory control of respiratory pumping in *Aplysia californica*. J. Exp. Biol. **117**: 15 - 27.

Dale, B. (1974). Extrusion, retraction and respiratory movements in *Helix pomatia* in relation to distribution and circulation of the blood. J. Zool., Lond. **173**: 427 - 439.

Daxboeck, C. and Holeton, G. F. (1978). Oxygen receptors in the rainbow trout, *Salmo gairdneri*. Can. J. Zool. **56**: 1254 - 1259.

Dejours, P. (1981). Principles of comparative respiratory physiology. Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford.

Eberly, L., Kanz, J., Taylor, C. and Pinsker, H. (1981). Environmental modulation of a central pattern generator in freely behaving *Aplysia*. Behavioral and Neural Biology **32**: 21 - 34.

de Fur, P and K. Lukowiak (1982). Some *in vivo*- and *in vitro* characteristics of *Aplysia californica* haemolymph. J. Exp. Biol. **101**: 347 - 351.

Janse, C. (1981). The effect of oxygen on gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **142 A**: 51 - 59.

Johansen, K. (1970). Air breathing in fishes. In: W.S. Hoar and D.J. Randall (Eds.), Fish Physiology, vol. IV . Academic Press, New York.

Jones, J. D. (1961). Aspects of respiration in *Planorbis corneus* L. and *Lymnaea stagnalis* L. (Gastropoda: Pulmonata). Comp. Biochem. Physiol. **4**: 1 - 29.

Koester, J., Dieringer, N. and Mandelbaum, D. E. (1979). Cellular neuronal control of molluscan heart. Am. Zool. **19**: 103 -116.

Kupfermann, I. and Kandel, E. R. (1969). Neuronal controls of a behavioral response mediated by the abdominal ganglion of *Aplysia*. Science **164**: 847 - 850.

Levy, M., Achituv, Y., and Susswein, A. J. (1989). Relationship between respiratory pumping and oxygen consumption in *Aplysia depilans* and *Aplysia fasciata*. J. Exp. Biol. **141**: 389 - 405.

Massabuau, J. C. and Burtin, B. (1984). Regulation of oxygen consumption in the crayfish *Astacus leptodactylus* at different levels of oxygenation: role of peripheral O_2 chemoreception. J. Comp. Physiol. **155 B**: 43 - 49.

Mc Mahon, R. F. (1983). Physiological ecology of freshwater pulmonates. In: W. D. Russel-Hunter (Ed.), The Mollusca, Vol. 6: Ecology . Academic Press, New York.

Milsom, W. K. (1991). Intermittent breathing in vertebrates. Annu. Rev. Physiol. **53**: 87 - 105.

O'Regan, R. G. and Majcherczyk, S. (1982). Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. J. Exp. Biol. **100**: 23 - 40.

Piiper, J. (1982). Respiratory gas exchange at lungs, gills and tissues: mechanisms and adjustments. J. Exp. Biol. **100**: 5 - 22.

Shelton, G. and Boutilier, R. G. (1982). Apnoea in amphibians and reptiles. J. Exp. Biol. **100**: 245 - 273.

Shelton, G., Jones, D. R. and Milsom, W. K. (1986). Control of breathing in ectothermic vertebrates. In: A. P. Fishman, N. S. Cherniack and J. G. Widdicombe (Eds.), Handbook of Physiology, section 3: The respiratory system, Volume II, Control of breathing, part 2. Amer. Physiol. Soc., Bethesda.

van der Steen, W. J., van den Hoven, N. P. and Jager, J. C. (1969). A method for breeding and studying freshwater snails under continuous water change, with some remarks on growth and reproduction in *Lymnaea stagnalis* (L.). Neth. J. Zool. **19**: 131-139.

Taylor, E.W.(1982).Control and co-ordination of ventilation and circulation in crustaceans: responses to hypoxia and exercise. J. Exp. Biol. **100**: 289 - 319.

Truchot, J. P. (1990). Respiratory and ionic regulation in invertebrates exposed to both water and air. Annu. Rev. Physiol. **52**: 61 - 76.

CHAPTER 3

RELATIONSHIP BETWEEN HAEMOLYMPH PO_2, RESPIRATORY BEHAVIOUR, HEART RATE AND ANAEROBIC METABOLISM, IN THE FRESHWATER PULMONATE SNAIL *LYMNAEA STAGNALIS*

SUMMARY

Specimens of the bimodal breathing snail, *Lymnaea stagnalis*, were subjected to forced submergence of varying duration in either O_2 rich- or O_2 poor water. During forced submergence in O_2 rich water, haemolymph PO_2 gradually decreases; in spite of this, no change in heart rate occurs, nor does anaerobiosis occur. The animals become more inclined to move towards the water surface, however, and tend to breathe slightly longer, once they are allowed to surface.

During submergence in O_2 poor water, haemolymph PO_2 decreases more rapidly and to a lower steady state value than during submergence in O_2 rich water. The animals show a stronger tendency to move towards the water surface. In addition, metabolism becomes anaerobic, heart rate decreases and a considerable increase of the duration of lung ventilation occurs during the surfacing period following the forced submergence.

It is concluded that *Lymnaea* is relatively tolerant to changes in internal PO_2, that are probably inherent to its bimodal mode of breathing. There exists, however, a critical level of internal PO_2, below which the animal responds with a change in metabolism and heart rate, and a long lasting change in respiratory behaviour. The latter persists long after PO_2 of haemolymph, lung cavity and ambient water have been restored. The results support our previous conclusion, that probably both internal and external O_2 chemosensitivity is involved in control of respiratory behaviour.

INTRODUCTION

In the previous chapter, we presented a description of the different components of ventilatory behaviour of *Lymnaea* and of the factors that control this behaviour. We concluded that low ambient PO_2 as well as high ambient PCO_2 affect migratory behaviour (resulting in a decerase of the non-ventilatory intervals between successive bouts of aerial ventilation) and ventilatory behaviour at the surface (resulting in an increase of the duration of the ventilatory period through an increase of number or duration of individual

pneumostome movements, respectively). During these conditions the changes in ambient PO_2 and ambient PCO_2 will have resulted in changes in internal respiratory gas tensions (that is, in lung cavity and circulatory system). Therefore, these observations do not allow to distinguish between behavioural changes that result from changes in external (water) or internal (lung cavity or circulatory system) PO_2 or PCO_2.

To explore this aspect further, the effects of low ambient PO_2 (but not high ambient PCO_2) were studied by changing independently the PO_2 of the ambient water or the PO_2 of the inspired air. At low PO_2 of the ambient water, the animals maintained normoxic haemolymph PO_2. In contrast, at low PO_2 of the inspired air, haemolymph PO_2 was decreased.

Since non-ventilatory intervals between successive bouts of aerial ventilation were decreased at low PO_2 of the ambient water, but not at low PO_2 of the inspired air, we concluded that this migratory behaviour is primarily affected by the PO_2 of the ambient water. Or, in other words: the tendency of the animals to orientate and move towards the water surface in order to breathe air is to an important extent determined by the PO_2 of the ambient water.

Similarly, since at the water surface ventilatory movements of the pneumostome were affected at low PO_2 of the inspired air, but not at low PO_2 of the ambient water, we concluded that the tendency of the animal to remain at the water surface and carry out ventilatory movements was primarily affected by the PO_2 in the lung cavity (or the circulatory system in the vicinity of the lung cavity).

The data of the preceding chapter gave a first clue as to what factors impinge upon different components of ventilatory behaviour in *Lymnaea*. What is lacking, however, is information of the dynamics how exactly environmental changes in PO_2 (and PCO_2, which will not, however, be pursued here any further) affect the PO_2 in the haemolymph and the lung cavity. The bimodal mode of breathing, as displayed by *Lymnaea*, has been demonstrated to result in relatively large fluctuations of internal gas tensions in other bimodal breathing species (Shelton and Croghan, 1988; Milsom, 1991): The internal PO_2 is maximal during aerial ventilation, and gradually decreases during the subsequent submergence. The extent of these fluctuations depends primarily on the capacity of the cutaneous gas exchanger and the PO_2 of the ambient water. These fluctuations in internal respiratory gas tensions that are reported to be associated with bimodal breathing, raise interesting questions with respect to control of ventilatory behaviour: on the one hand, bimodal breathers should be sufficiently responsive to internal or external changes in PO_2, in order to adjust their behaviour to ensure adequate oxygention of the tissues. On the other hand, however, bimodal breathers should be relatively tolerant to fluctuations of internal gas tensions that are inherent to this mode of ventilation.

The purpose of the present chapter is to obtain some information as to how haemolymph PO_2 behaves after aerial ventilation has taken place during submergence of

various periods of time in O_2 rich- or O_2 poor water. In parallel experiments, we examined whether during these periods of submergence changes take place in orientation of the animals with respect to the water surface and in heart rate. Heart rate has been demonstrated to be indicative of the respiratory condition of several invertebrates (see Herreid, 1980), and was known to be affected by the ambient PO_2 (De With and Van der Wilt, unpublished observations). Also, the concentration of D-lactate was measured in the haemolymph of snails kept submerged for various periods of time in O_2 rich or O_2 poor water, to determine whether anaerobiosis occurred under these conditions. Finally, the duration of ventilation was measured after the forced submergence, when the animals were allowed to breathe air at normoxic PO_2.

The data should provide some more insight into the physiology of the bimodal type of breathing of *Lymnaea*: does haemolymph PO_2 in *Lymnaea* change during submergence; does this depend on the PO_2 of the ambient water; what is the relative contribution of gas exchange across the skin; is there a relation between changes in haemolymph PO_2 and migratory behaviour, heart rate, onset of anaerobiosis and subsequent ventilatory behaviour at the surface; and, finally: do these observations of the intact animal allow to make inferences about localization of O_2 chemosensitivity and the processes that are mediated by it?

MATERIALS AND METHODS

Snails

Adult specimens of *Lymnaea stagnalis* (L.), shell length about 30 mm were used. They were bred under laboratory conditions at a 12 - 12 hr light - dark regimen and were fed lettuce ad libitum (van der Steen, van den Hoven and Jager, 1969).

Incubations

Snails were transferred from their breeding tanks to an aquarium with air - saturated water and allowed free access to the water surface. Immediately after voluntary lung ventilation, snails were kept submerged in either O_2 rich (150 Torr) or O_2 poor (25 Torr) water. This was achieved by transferring each snail to a perforated jar, placed in an aquarium (30 x 20 x 23 (h) cm), filled with either O_2 rich or O_2 poor water. A glass plate on top of each jar prevented the animals from surfacing. The water was gently gasified with the appropriate gas mixture (500 ml gas/minute). Gas mixtures were obtained by mixing pure O_2 and pure N_2 (Hoekloos, The Netherlands) with mass flow controllers (Brooks Instruments, model 5850 TR), connected to a Four Channel Control Unit (Brooks Instruments, model 5878). The PO_2 of the water was recorded continuously during the experiment. During the forced submergence snails were allowed to feed on lettuce.

Haemolymph PO_2 and heart rate were determined after keeping the animals submerged during 0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes. The observations at t_0 were in fact determined after 30 sec of submergence. Duration of lung ventilation was determined in animals during surfacing after forced submergence of 5, 10, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes. Gravity orientation was determined immediately after lung ventilation and after submergence of the animals during 5, 10, 15, 30, 45 and 60 min. Haemolymph D-lactate, indicative for anaerobic metabolism (Wijsman *et al.*, 1985), was determined in animals after submergence of 5, 10, 15, 30, 45, 60 and 120 minutes in O_2 poor water and of 30, 60 and 240 minutes in O_2 rich water. For each determination, 5 measurements were done on different snails. Each snail was used for one measurement only. All experiments were carried out at room temperature.

Determinations

PO_2 of the water and of the haemolymph and heart rate were determined as described in the previous chapter. Gravity orientation was determined by carefully allowing the snails to attach to the vertical side of an aquarium (size 30 x 20 x 23 (h) cm), in the centre of a circle, 9 cm in diameter. The snails were always allowed to attach in the same position, with their longitudinal axis perpendicular to the direction of gravity and their head facing to the right relative to the observer. Creeping tracks were copied and crossing positions (degrees) of the creeping tracks with the circle were subsequently determined for each snail. A negative value of the crossing position (crossing positions on the upper half of the circle) represents negative geotaxis of the animal. Duration of lung ventilation after submergence was determined during the subsequent surfacing period. Number and duration of pneumostome opening movements which occurred, before the snails started a subsequent submergence were measured. When more than one pneumostome opening occurred, total duration of lung ventilation was derived from summation of the durations of individual opening movements. The concentration of D-lactate in the haemolymph, collected from the hemal pore was measured as described by Wijsman *et al.* (1985).

Statistical analysis

The experimental design is factorial with two factors: PO_2 of the water and duration of the submergence. Analysis of variance was used to analyze all measurements. We applied non-linear regression analysis to the data of the haemolymph PO_2. Exponential curves were numerically fitted to these data with the method of maximum likelihood estimation (Hogg and Craig, 1978). Differences between curves were tested with generalized likelihood-ratio tests (Rao, 1973).

RESULTS

After voluntary aerial ventilation, snails were forced to remain submerged in either O_2 rich or O_2 poor water. The effect of this treatment on haemolymph PO_2 was studied by selecting snails after different time intervals; haemolymph was collected from these animals and haemolymph PO_2 was measured. For each measurement a different snail was used. In separate experiments, the effects of such forced submergence in either O_2 rich or O_2 poor water were studied on haemolymph D-lactate, heart rate, orientation with respect to the water surface, and duration of lung ventilation after the forced submergence. The results are shown in Table 1. The values represent means and standard deviations of measurements on 5 different snails. Analysis of variance shows that there were statistically significant interactions between the effects of the duration of the forced submergence and the PO_2 of the water with respect to all the parameters. In the following, these effects will be described, referring to Figures 1 A - E, which are the graphic presentations of the data of Table 1.

Effects on haemolymph PO_2

Fig. 1 A shows that in O_2 rich- as well as in O_2 poor water, the PO_2 of the haemolymph decreased immediately after the onset of the forced submergence.In O_2 rich water the PO_2 of the haemolymph decreased during submergences of up to about 60 min. During submergences of more than 60 min, haemolymph PO_2 remained approximately constant. During submergences in O_2 poor water too, the PO_2 of the haemolymph initially decreased and subsequently remained approximately unchanged. The final level was, however, lower as compared to submergences in O_2 rich water. Also, the change appeared to occur more rapidly.

In the following it was determined whether the rate of change and the final level of the haemolymph PO_2 were significantly different from each other during forced submergences in O_2 rich- and O_2 poor water. For this purpose, the data were used to describe the change in haemolymph PO_2 during forced submergence as a decreasing exponential function, with the general formula

$$P(t) = P_\infty + (P_0 - P_\infty)e^{-at}$$

P_∞ represents the stable value of haemolymph PO_2, reached after prolonged submergence in O_2 rich or O_2 poor water (steady state value). P_0 represents the value of haemolymph PO_2 at the beginning of the submergence (initial value). a represents the rate of decrease of haemolymph PO_2 (time constant). Several possibilities were studied: one model function for both data sets (3 parameters to be estimated), or two model functions with some or all parameters different (4 to 6 parameters to be estimated). The results are presented in Table 2. For each possibility the residual sum of squares was calculated.

Subsequently, it was determined whether the addition of an extra parameter rendered a significantly better fit of the data.

From Table 2 it follows that the 5 parameter case (P_0 equal for both data sets) gave the best fit. It was significantly better than the 4 parameter case ($p < 0.001$). The 6 parameter case showed hardly any improvement. Fig. 2 shows the mean values and the two curves (5 parameter case).

This implies that, as expected, no significant difference existed between haemolymph PO_2 values, measured in snails immediately after the onset of the forced submergence in O_2 rich- or in O_2 poor water (P_0). Haemolymph PO_2 of snails decreased significantly faster (a) and to a significantly lower value (P_∞) during forced submergence in O_2 poor water as compared to forced submergence in O_2 rich water. In addition, using the model functions of the fitted curves, it can be calculated that after forced submergences of approximately 20 minutes, haemolymph PO_2 of snails in O_2 poor water dropped below the value which was finally reached in snails in O_2 rich water.

Effects on haemolymph D- lactate

Fig.1 B shows that during forced submergences in O_2 poor water, the amount of D-lactate increased strongly, starting at 15 to 30 min after the onset of the submergence. In contrast, the amount of D-lactate in the haemolymph did not change during forced submergences in O_2 rich water.

Effects on heart rate

Fig. 1 C shows that during forced submergences in O_2 rich water, heart rate did not change. Forced submergences in O_2 poor water resulted in a considerable decrease of heart rate. The decrease started at 15 - 30 minutes after the onset of forced subemergence, continued during submergences of up to 150 min and subsequently remained unchanged.

Effects on lung ventilation

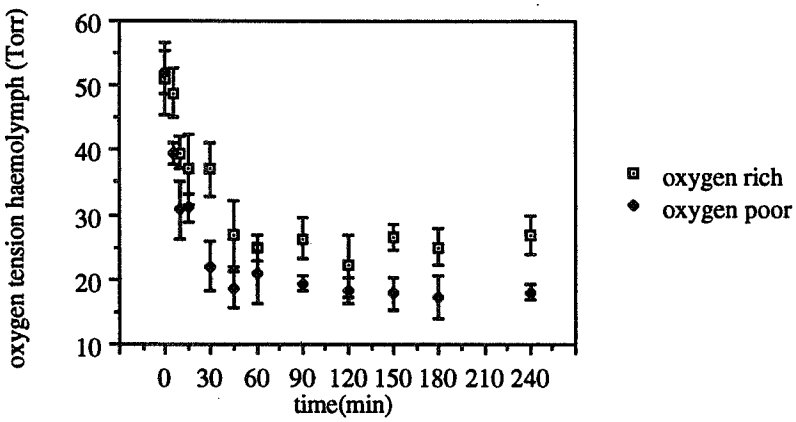
Fig. 1 D shows that submergences in O_2 poor water resulted in a considerable increase of the duration of lung ventilation which started at 15 to 30 min after the beginning of the submergence and continued for the remaining durations of submergence. After 240 min

---

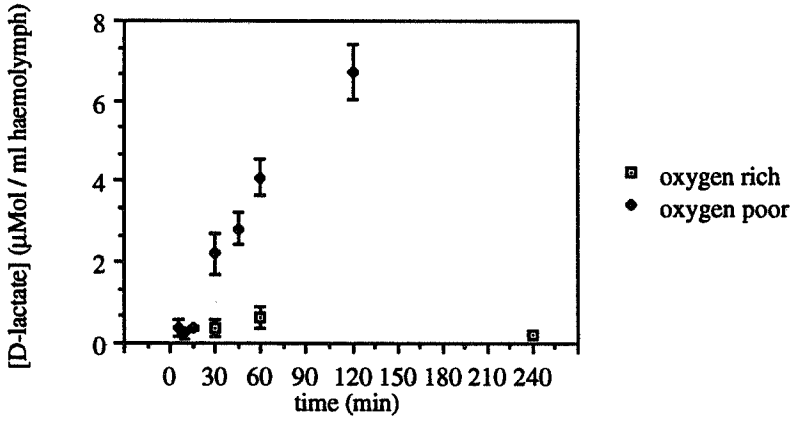
Table 1. Effects of forced submergence of various durations (up to 240 min) in either O_2 rich - or O_2 poor water on haemolymph PO_2 (Torr), heart rate (beats per min), concentration D-lactate in the haemolymph (µMol / ml), duration of ventilation after the forced submergence (min) and orientation. In the latter case, the mean crossing positions (degrees) are presented; crossing positions near 90^0 signify vertical movement in the direction of the water surface, crossing positions near 0^0 signify horizontal movement, crossing positions near -90^0 signify vertical movement in the direction of the bottom of the water.

Table 1.

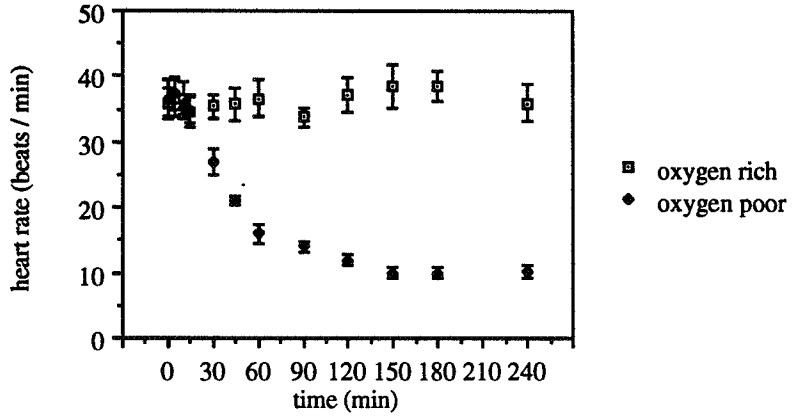
		DURATION OF DIVE (MIN)											
PARAMETER:	P_{O_2} water	0	5	10	15	30	45	60	90	120	150	180	240
P_{O_2}	normal	51.10 ±5.70	48.80 ±3.84	39.60 ±2.49	37.06 ±5.51	37.00 ±4.29	27.00 ±5.10	25.03 ±2.07	26.38 ±3.14	22.16 ±4.84	26.54 ±2.07	24.98 ±2.75	26.90 ±2.86
	low	52.00 ±3.20	39.48 ±1.50	30.74 ±4.41	31.04 ±2.12	22.02 ±3.89	18.55 ±2.70	20.84 ±4.60	19.41 ±1.23	18.28 ±2.02	17.82 ±2.37	17.32 ±3.28	17.98 ±1.15
heart rate	normal	36.0 ±2.12	36.6 ±2.88	36.4 ±2.70	34.6 ±2.30	35.4 ±1.82	35.8 ±2.49	36.6 ±2.88	33.8 ±1.48	37.2 ±2.59	38.6 ±3.29	38.4 ±2.30	36.0 ±2.92
	low	36.5 ±3.11	37.4 ±2.41	35.2 ±0.84	35.0 ±2.12	27.0 ±1.87	21.0 ±0.71	16.0 ±1.58	14.0 ±0.71	12.0 ±0.71	10.0 ±0.71	10.0 ±0.71	10.2 ±0.84
haemolymph D-lactate	normal					0.37 ±0.21		0.63 ±0.28					0.21 ±0.11
	low		0.37 ±0.21	0.25 ±0.12	0.37 ±0.06	2.20 ±0.51	2.83 ±0.39	4.10 ±0.43		6.71 ±0.69			
duration of ventilation	normal		0'40" ±0'06"	0'56" ±0'05"	1'03" ±0'15"	1'35" ±0'18"		1'39" ±0'28"	1'30" ±0'14"	1'18" ±0'18"	1'26" ±0'10"	1'23" ±0'13"	1'25" ±0'13"
	low		1'32" ±0'48"	1'40" ±0'41"	1'53" ±0'23"	5'55" ±0'56"		7'29" ±1'00"	9'21" ±0'21"	11'14" ±1'50	13'56" "±1'15"	17'58" ±2'36"	24'01" ±1'55"
crossing position	normal	-71.80 ±15.24	24.80 ±43.18	56.20 ±12.93	63.80 ±15.97	60.80 ±32.91	55.00 ±13.02	55.80 ±19.34					
	low	7.80 ±49.21	70.80 ±18.89	82.40 ±4.56	74.80 ±9.26	82.20 ±5.54	83.60 ±4.16	82.20 ±4.66					



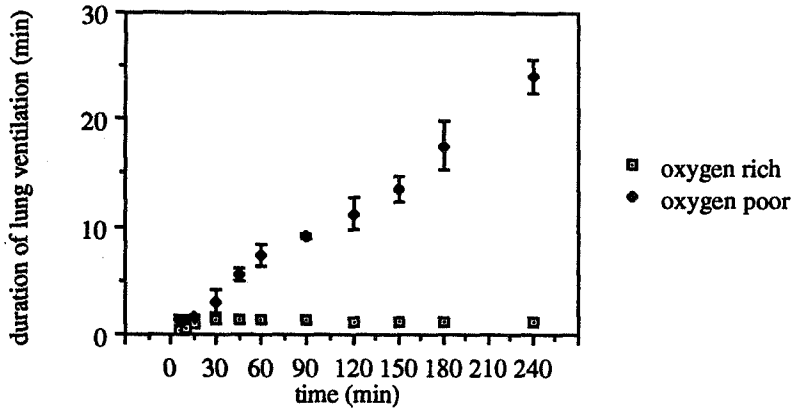
Figuur 1A



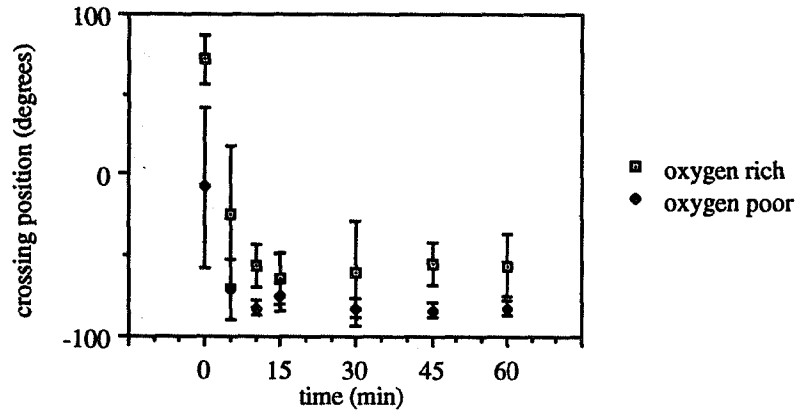
Figuur 1B



Figuur 1C



Figuur 1D



Figuur 1E

Fig. 1. Time course of changes in haemolymph PO_2 (Torr) (A), concentration D-lactate (µMol / ml haemolymph) (B), heart rate (beats. min-1) (C), duration of lung ventilation (D) and crossing position (E) after different periods of forced submergence in either normoxic or hypoxic water. Mean values and s.d. of five different observations.

of submergence in O_2 poor water, the duration of lung ventilation was 24' 01" ± 1' 55". During this period, numerous opening movements of the pneumostome occurred varying in duration from a few seconds to up to 2 min.

After forced submergences in O_2 rich water of up to 30 min the duration of lung ventilation slightly increased and subsequently remained approximately constant. The initial increase, which is very modest as compared to the increase which occurs in animals after forced submergence in O_2 poor water, roughly coincides with the decrease of the PO_2 of the haemolymph during this part of the forced submergence in O_2 rich water (Figure 3).

To determine whether the long lasting change in lung ventilation could be related to a slow recovery of the PO_2 of the haemolymph, the PO_2 of the haemolymph was measured 5 minutes after transferring the animals to O_2 rich water and allowing them to breathe atmospheric air. After a forced submergence of 4 hours in O_2 poor water, this treatment resulted in complete recovery of haemolymph PO_2 (mean 53.1 Torr, s.d. 2.3, n= 5). Likewise, heart rate was restored to approximately 34 beats per minute (mean 33.8, s.d. 1.8, n= 5), a value not significantly different from control values. Consequently, the long lasting surfacing and lung ventilation after such a prolonged submergence take place while the PO_2 in the haemolymph and the ambient water are restored to normoxic values.

Table 2. Results of curve fitting to the haemolymph PO_2 data with 3 to 6 parameters. S2 is the residual sum of squares. The 5 parameter case is shown in Fig. 2.

P_∞		P_0		a		S2	para-
O_2-rich	O_2-poor	O_2-rich	O_2-poor	O_2-rich	O_2-poor		meters
21.86		51.02		0.0597		3360.09	3
26.83	17.73	51.51		0.0641		1823.18	4
25.03	18.55	51.38		0.0435	0.0812	1581.64	5
25.04	18.54	51.52	51.19	0.0439	0.0805	1581.29	6

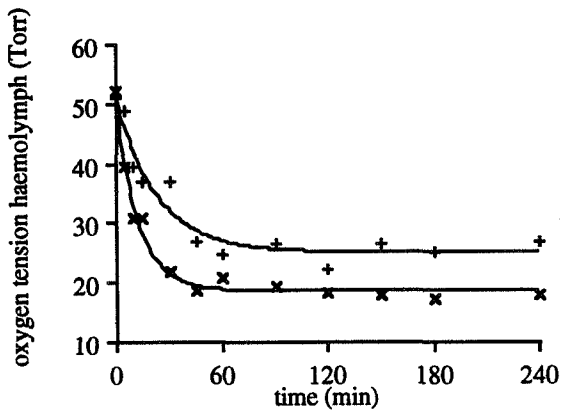


Fig. 2. Time course of changes in haemolymph PO_2 (Torr) after different periods of forced submergence in normoxic or hypoxic water. Fitted curves based on individual data (5 parameter case, see Table 2) and mean values of five different observations are displayed. +: mean values of PO_2 of the haemolymph of snails kept in O_2 rich water, x: mean values of PO_2 of the haemolymph of snails kept in O_2 poor water.

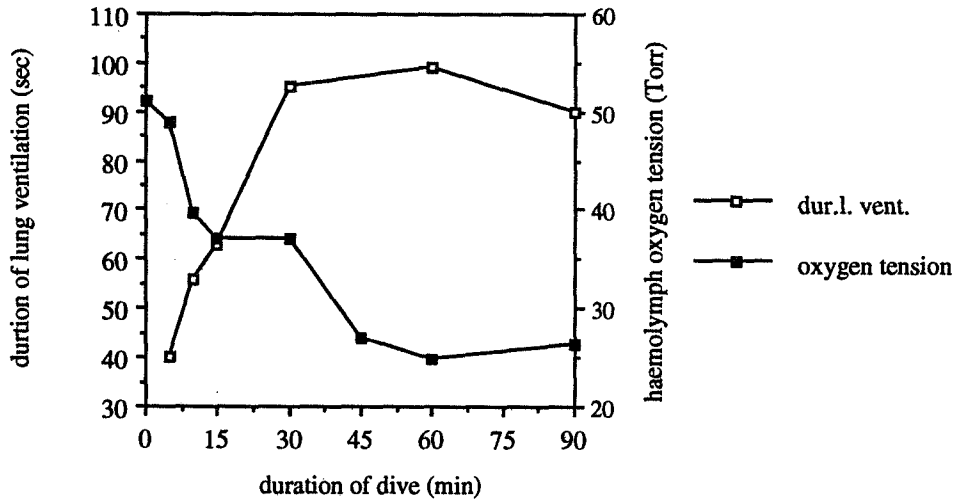


Fig. 3: Change in mean duration of lung ventilation after forced submergence of up to 90 min in O_2 rich water, and concomitant change of haemolymph PO_2 during this period of submergence.

Effects on orientation

Figs.1 E and 4 show that in O_2 rich water, immediately after lung ventilation all snails moved towards the bottom of the water. After a submergence of 5 min in O_2 rich water orientation varied considerably among individual snails; two out of five snails moved to the surface of the water. The other snails did not move clearly to either the bottom or the surface of the water. After a submergence of 10 min, all snails moved to the water surface. Submergences in O_2 rich water of periods of 15 min or more did not result in additional changes in orientation as compared to those of 10 min. All snails moved to the water surface, the variability among different individuals remained approximately equal with increasing durations of forced submergence.

In O_2 poor water, orientation among different snails varied considerably immediately after lung ventilation. One out of five snails moved to the surface, while the other four did not clearly move to either the bottom or the surface of the water. A submergence of 5 min in O_2 poor water resulted in movement to the surface in all snails. After submergences of 10 min or more in O_2 poor water, all snails made straight creeping tracks to the water surface, crossing the arena near -90°.

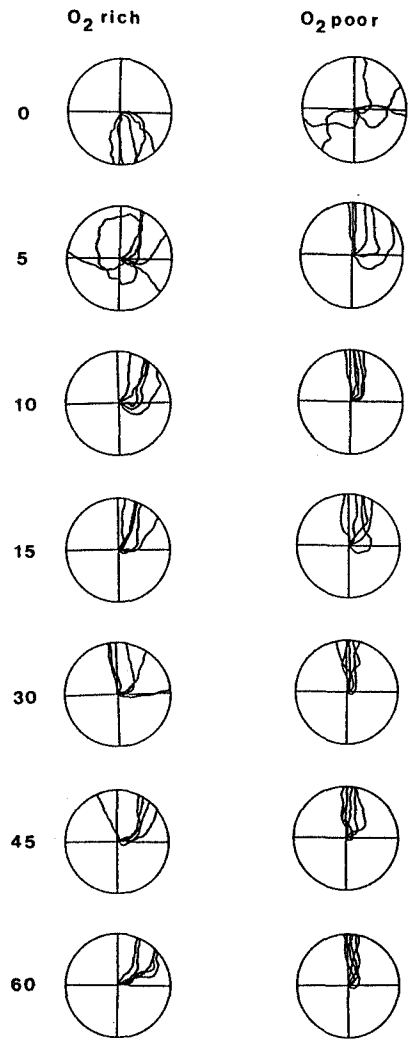


Fig. 4. Creeping tracks of snails after different periods of forced submergence in normoxic or hypoxic water. The geotaxis test was done in water with the same PO_2 as the water in which the snails were kept submerged.

DISCUSSION

The present study shows that during submergence in O_2 rich water, as well as during submergence in O_2 poor water, haemolymph PO_2 in *Lymnaea* decreases. It also shows that during submergence in O_2 rich water, in spite of the decrease of haemolymph PO_2,

heart rate does not change, nor is there any sign of anaerobic metabolism (accumulation of acidic end-products of anaerobiosis in the haemolymph). This suggests that *Lymnaea* tolerates these internal PO_2 changes relatively well. During submergence in O_2 rich water the tendency of the animals to move to the water surface increases; at the surface, the duration of ventilation after prolonged forced submergence is slightly increased.

In contrast, during submergence in O_2 poor water *Lymnaea* showed a decrease in heart rate and a switch to anaerobiosis; also, there was a considerable increase of the duration of ventilation after forced submergence. These changes coincided with the decrease of the haemolymph PO_2 below the steady-state level that was reached during submergence in O_2 rich water. This suggests that there exists a critical level of internal PO_2: as long as the internal PO_2 remains above this level, *Lymnaea* responds to a decrease in internal O_2 by an increased tendency to move to the water surface and a slight increase of the duration of lung ventilation. When the internal PO_2 decreases below this level, a much more comprehensive response occurs, involving adjustment of heart rate and metabolism as well. This response is probably aimed at a reduction of energy expenditure and anaerobic ATP production (see Herreid, 1980).

Under laboratory conditions, the mean duration of voluntary dives in well oxygenated water is approximately 9 minutes (see chapter 2). The present data show that during submergences of this duration a decrease of haemolymph PO_2 occurs. Therefore, during voluntary dives fluctuations of haemolymph PO_2 will occur; Table 1 shows that after a period of submergence of 10 minutes in O_2 rich water, haemolymph PO_2 has decreased from 51.1 to 39.6 Torr.

Decrease of haemolymph PO_2 during submergence probably reflects different relative contributions of lung- and skin respiration to total gas exchange

The time course of the change in haemolymph PO_2 during submergence in O_2 rich water closely resembles that of the decrease of the PO_2 of the lung cavity in *Lymnaea* as measured by Precht (1939). Precht' s data show that during submergence, the O_2 content of the lung cavity is gradually depleted. As this is accompanied by a decrease of the haemolymph PO_2, it can be inferred that O_2 uptake through the skin cannot fully compensate for the decrease in O_2 diffusion from the lung cavity into the haemolymph. A higher diffusion rate accross the lung epithelium as compared to the skin could be due to the fact that the lung epithelium is highly vascularized and much thinner than the skin (Bekius, 1972). This would imply that the intrinsic gas diffusion capacity of the lung epithelium exceeds that of the skin. As soon as the haemolymph PO_2 decreases, O_2 diffusion through the skin will slightly increase, as a result of the increased PO_2 gradient. Both processes will proceed, untill a new equilibrium is reached. During this equilibrium, O_2 consumption will be equal to the amount of O_2 diffusing from the water into the haemolymph.

In the case of forced submergences in O_2 poor water too, the decrease of the PO_2 of the haemolymph is similar to the decrease of the PO_2 in the lung cavity (Precht, 1939). The lower steady state level and the more rapid decrease as compared to the changes observed during submergence in O_2 rich water can be explained by the considerable decrease of the contribution of skin respiration.

Changes in lung ventilation, metabolism, heart rate and gravity orientation in relation to changes in PO_2 in lung cavity and haemolymph and PO_2 of the ambient water

In contrast to the approach of the preceding chapter, in the present study the time course of changes in ventilatory behaviour has been established, together with the time course of changes in haemolymph PO_2. In the following, it will be discussed whether further conclusions can be drawn from the data with respect to the nature and localization sensory structures, mediating these changes.

The increase of the duration of lung ventilation which is oserved after submergences in O_2 rich water of up to 30 min, coincides with the decrease of haemolymph PO_2 and postulated decrease of the PO_2 in the lung cavity (see Fig. 3). It is conceivable that in these instances it takes proportionally more time to restore the PO_2 in either or both compartments. Similarly, the consistently lower PO_2 in the haemolymph (and lung cavity) of animals that were kept submerged in O_2 poor water might account for the observation that lung ventilation invariably lasts longer as compared to lung ventilation after submergence of similar duration in O_2 rich water.

The onset of the long-lasting duration of ventilation following submergence in O_2 poor water is clearly related to the decrease of the PO_2 below the steady state level, reached during submergence in O_2 rich water. This decrease of haemolymph PO_2 is probably responsible for the onset of anaerobiosis. The concomitant decrease in heart rate may also be related to this change in internal PO_2. In addition, however, it may be due to accumulation of acidic end products of anaerobiosis or to the resulting changes in internal pH. This probably holds for the considerable increase of the duration of lung ventilation which was observed after prolonged submergence in O_2 poor water. This increased duration of lung ventilation continues, even after the PO_2 of the haemolymph was already restored. This phenomenon may occur to meet the increased demand for O_2, which occurs in *Lymnaea* after a period of anaerobiosis, when normoxic conditions are restored (von Brand and Mehlman, 1953). It is important to note, that respiratory movements in this condition differ from those, observed when *Lymnaea* is forced to breathe air at low PO_2 (see Chapter 2): when breathing air at low PO_2, short lasting pneumostome movements are carried out at high frequency. This was *not* observed when the animals were allowed to breathe normoxic air after the period of forced submergence in O_2 poor water. In both cases, the internal pH is probably affected by the onset of anaerobiosis. Thus, a change in internal pH alone cannot account for the change in respiratory

movements, observed during hypoxia, and should probably be attributed to the low PO_2 of the inspired air.

With respect to orientation to the water surface, the data show that immediately after lung ventilation, the orientation of snails in O_2 rich water and of snails in O_2 poor water are clearly different from each other. Haemolymph PO_2 of these snails in the two conditions (that is, immediately after aerial ventilation) was the same. Therefore, this difference in orientation must be due to the difference in PO_2 of the water. This is in keeping with the results of the previous chapter, and with earlier observations, made by Janse (1981), which indicated the presence of sensory structures involved in reception of the PO_2 of the water, affecting orientation with respect to the water surface.

In addition, our data show that orientation of the animals during submergence in O_2 rich water changed: the tendency to orientate to the water surface progressively increased. This too, is consistent with earlier observations made by Janse (1981). The change in orientation of the animals after about 10 minutes is in keeping with the observation that in freely behaving snails the mean duration of dives in O_2 rich water is approximately 9 min (chapter 2). Clearly, this change in orientation can *not* be mediated by external O_2 chemosensitivity, since the PO_2 of the ambient water remained unchanged. Several possibilities exist for the sensory mechanisms, mediating this change in orientation:

It may be due to changes of the PO_2 in either the lung cavity or the haemolymph that occur during submergence. In this respect, it is not possible to distinguish between these two compartments, since the time course of the change in PO_2 in the lung cavity and in the haemolymph are very similar (Precht, 1939). In addition to possible chemosensitive effects, the change in orientation may be mediated by mechanoreceptors, sensitive to changes in buoyancy of that part of the body, containing the lung cavity (Janse, 1982), or mechanoreceptors, sensitive to changes in lung volume (Jones, 1961).

In sum, the present observations confirm the conclusion of the previous chapter, that orientation of *Lymnaea* with respect to the water surface is affected by the PO_2 of the ambient water. In addition, however, this orientation is affected by either internal PO_2 changes or by a mechanosensory mediated process. With respect to control of ventilatory movements at the water surface, the present data indicate that in addition to the effects of PO_2 and PCO_2 of the inspired air (chapter 2), these are affected by the presence of acidic end-products of anaerobiosis in the haemolymph or the associated change in haemolymph pH. This phenomenon is reminiscent of what has been reported about control of pneumostome movements in the terrestrial slaug, Limax maximus (Dickinson et al., 1988). In this animal, pneumostome movements are affected by the osmolality of the haemolymph, which in turn is affected by the degree of dehydration. This effect appears to be partly mediated by peptide hormones. The contribution of chemosensitive structures at multiple sites to adjustments of ventilatory behaviour is compatible with observations in vertebrates. Here, changes in blood PO_2 or PCO_2 mediate instantaneous adjustments of

respiration and circulation, whereas changes in PO_2, PCO_2 or pH in the brain fluid mediate long term adjustments of these functions (O'Regan and Majcherczyk, 1982).

REFERENCES

Bekius, R. (1972). The circulatory system of *Lymnaea stagnalis* (L.). Neth. J. of Zool. **22** (1): 1 - 58.

von Brand, Th. and Mehlman, B. (1953). Relations between pre- and post-anaerobic oxygen consumption and oxygen tension in some freshwater snails. Biol. Bull. **104**: 301-312.

Dickinson P. S., Prior D. and Avery C (1988). The pneumostome rhythm in slugs: a response to dehydration controlled by hemolymph osmolality and peptide hormones. Comp. Biochem. Physiol. **89A** (4): 579 - 585.

Herreid, C.F. (1980). Hypoxia in invertebrates. Comp. Biochem. Physiol. **67A**: 311 - 320.

Hogg, R.V. and Craig, A.T. (1978). Introduction to Mathematical Statistics. Macmillan, New York.

Janse, C. (1981). The effect of oxygen on gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **142**: 51 - 59.

Janse, C. (1982). Sensory systems involved in gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **145**: 311 - 319.

Jones, J.D.(1961). Aspects of respiration in *Planorbis corneus* L. and *Lymnaea stagnalis* L. (Gastropoda:Pulmonata). Comp. Biochem. Physiol. **4**: 1 - 29.

Milsom W. K. (1991). Intermittent breathing in vertebrates. Annu. Rev. Physiol. **53**: 87 - 105.

O'Regan, R.G. and Majcherczyk, S. (1982). Role of peripheral chemoreceptors and central chemosensitivity in the regulation of respiration and circulation. J. Exp. Biol. **100**: 23 - 40.

Precht, H. (1939). Die Lungenatmung der Süsswasserpulmonaten (zugleich ein Beitrag zur Temperaturabhängigkeit der Atmung). Z. für vergleichende Physiol. **26**: 696 - 739.

Rao, C.R. (1973). Linear Statistical Inference and its Applications, 2nd edn. Wiley, New York.

Shelton, G. and Croghan, P. C. (1988). Gas exchange and its control in non-steady-state systems: the consequences of evolution from water to air breathing in the vertebrates. Can. J. Zool. **66**: 109 - 123.

van der Steen, W. J., van den Hoven, N. P. and Jager, J. C. (1969). A method for breeding and studying freshwater snails under continuous water change, with some remarks on growth and reproduction in *Lymnaea stagnalis* (L.). Neth. J. Zool.**19**: 131-139.

Wijsman, Th. C. M., Van der Lugt, H. C. and Hoogland, H. P. (1985). Anaerobic metabolism in the freshwater snail *Lymnaea stagnalis*: haemolymph as a reservoir of D-lactate and succinate. Comp. Biochem. Physiol. **81B**: 889-895.

CHAPTER 4

SUPPRESSION OF RESPIRATORY BEHAVIOUR DURING EGG LAYING IN THE FRESHWATER PULMONATE SNAIL *LYMNAEA STAGNALIS*

SUMMARY

In the fresh water pulmonate *Lymnaea*, periods of aerial ventilation during surfacing are alternated by periods of submergence (non-ventilatory period). Egg laying requires that the snail remains submerged for a period which exceeds the normal duration of the non-ventilatory period. This suggests that the tendency to move towards the water surface in order to breathe, normally displayed by the animal, is suppressed during egg laying. To study the interactions between the two types of behaviour in more detail, we presented stimuli that normally induce immediate surfacing and aerial ventilation to specimens of *Lymnaea* during different phases of egg laying behaviour. We found that surfacing is suppressed during a period ranging from approximately 20 min prior to the initiation of ovipostion up to the termination of oviposition. This is only a fraction of the period that makes up the entire egg laying behaviour. We conclude that, although the appetetive and consummatory phase of egg laying are normally only rarely interrupted by aerial ventilation, *Lymnaea* can respond by surfacing and subsequent ventilation to surfacing stimuli during the major part of the appetetive phase, and ignores these stimuli only during the last part of the appetetive phase and during the consummatory phase of egg laying. With respect to possible underlying neural mechanisms, we conclude that suppression of surfacing is probably not due to the direct action of neuroactive peptides, responsible for the initiation of egg laying. Rather, the suppression of surfacing is likely to be mediated by sensory structures that are sensitive to the transport of eggs in the reproductive tract.

INTRODUCTION

The preceding chapters showed that ventilatory behaviour in *Lymnaea* involves movement of the animal to the water surface, opening- and closure movements of the pneumostome at the water surface and the subsequent return of the animal to the lower depths of the water. Changes in ambient PO_2 or PCO_2, and, probably, haemolymph pH

are major factors that control this behaviour. It appeared, for instance, that in O_2 rich water, surfacing followed by ventilation of the lung cavity, occurs approximately every 10 minutes; these non-ventilatory intervals were shortened during hypoxia and during hypercapnia. The picture that emerges from these data, then, is that a basic pattern of surfacing and submergence is generated in *Lymnaea*, mainly driven by O_2-, CO_2- or pH chemosensory drive. What is lacking in these experiments is interaction of ventilatory behaviour with other types of behaviour. Ventilatory behaviour was studied isolated from other types of behaviour. Ventilatory behaviour of the animals was not disturbed by potentially interfering factors such as the presence of preadtors or con-specifics or the necessity to feed on the bottom of the water. Particularly the bimodal mode of breathing of *Lymnaea* is likely to interfere with other types of activities. Regular excursions of *Lymnaea* to the water surface prohibit prolonged submergence, which may be necessary to carry out other types of behaviour. Thus, either the behaviour, displayed during submergence is interrupted in order to surface, or surfacing is suppressed in order to remain submerged for prolonged periods of time. The interaction between movement of the animal to the water surface in order to breathe, and under-water activities is a general feature of bimodal breathers (Boutilier, 1988).

Conceptually, observations on interactions between different types of behaviour has given rise to the idea of 'behavioural hierarchies'. When presented with different stimuli simultaneously, capable of eliciting conflicting types of behaviour, an animal will have to choose. Some types of behaviour are likely to take precedence over others; such observations on behavioural hierarchies have been related to the natural environment of the species and the presumed adaptive value to the animal. These phenomena have been extensively studied in molluscs, including *Aplysia* (Ziv *et al.*, 1991 a and b) and *Helix* (Adamo and Chase, 1991 a and b). In the two chapters that follow, interaction between ventilatory behaviour and two other types of behaviour (egg-laying and feeding, respectively) will be studied.

The present paper deals with interactions between surfacing and egg laying in *Lymnaea*, a behaviour which requires prolonged submergence. Egg laying in *Lymnaea* is a relatively stereotyped behaviour which consists of three separate phases. The first two phases, resting and turning, are appetetive phases which precede the third, consummatory phase, oviposition (ter Maat *et al.*, 1989). Egg laying can be elicited by transferring snails, that have been kept in dirty water for several days, to clean water (clean water stimulus, CWS) (ter Maat *et al.*, 1983). Egg laying is initiated by the discharge of the neurosecretory caudodorsal cells (CDCs), which release an ovulatory hormone and a number of other peptides (for review see Geraerts, ter Maat and Vreugdenhil, 1988). The time between the initiation of the discharge and the end of oviposition typically amounts approximately 110 min (ter Maat *et al.*, 1989). Our preliminary observations suggested that egg laying behaviour is only rarely interrupted by surfacing. This indicates that

respiratory behaviour in *Lymnaea* is at least partly suppressed during egg laying behaviour.

In order to study interactions between respiratory behaviour and egg laying in more detail, the response of the snail to exposure to air with low PO_2, a stimulus which normally induces immediate surfacing, was studied in snails, in which egg laying was induced by a CWS. The results show that in these animals the surfacing response is suppressed during oviposition and during a short period preceding oviposition. This shows that the response of *Lymnaea* is not rigid, allowing for prolonged submergence if so required.

MATERIALS AND METHODS

Animals

Adult specimens of the freshwater pulmonate snail, *Lymnaea stagnalis* were used with a shell length of approximately 30 mm and bred under standard laboratory conditions (van der Steen *et al.* 1969). The snails were fed lettuce every other day and kept at a light / dark regimen of 16 h light, 8 h dark (long day conditions).

Induction of egg laying

5 Days before the start of the experiment, snails were transferred to closed jars. Each jar contained 4 snails. During this period, the snails were fed lettuce ad libitum. A total number of 28 snails was used. At day 5, the snails were placed individually in clean jars, containing clean aereated tap water (20 ^{o}C) (CWS). This treatment is known to induce egg laying (ter Maat *et al.*, 1983).

Induction of surfacing

The procedure, used to induce surfacing of the snails was as follows. At variable time intervals after the CWS, the water in the jars containing the snails was removed with vacuum to such an extent that the water level was just beneath the snails. Subsequently, N_2 with a flow of 250 ml gas.min-1 (gas flow controller, Brooks Instruments) was flushed for 5 min into the space containing the snail. The PO_2 in the compartment thus decreased from 150 to approximately 15 Torr within 30 sec (as measured with polarographic O_2 electrode, Instech Laboratories). During this treatment, the snails always opened their pneumostome, thereby exposing the lung cavity to the atmosphere. Although this was not measured, the PO_2 in the lung cavity can be expected to decrease considerably as a result of gas exchange. After 5 min, water was added to the jar to restore the original water level. Our preliminary experiments showed that in animals, not involved in egg laying behaviour, this treatment induced surfacing of snails, followed by air breathing within a very short period of time (usually less than 5 min). This treatment

which was used to induce surfacing will be referred to in the folowing as *surfacing stimulus*. In the cases that the CWS resulted in egg laying, usually 3 surfacing stimuli were delivered to an animal before the start of oviposition.

Determinations

At different time intervals after the CWS, the latency between the termination of the surfacing stimulus (elevation of the water level) and the moment the snail reached the water surface and opened its pneumostome, was determined (surfacing latency). In addition, the initiation and duration of oviposition were determined, and the number of eggs was counted in each egg mass. As demonstrated by Ter Maat *et al.* (1989), a positive linear relationship exists between the number of eggs in an egg mass on the one hand and the latency between the CWS and the start of the oviposition on the other hand. Thus, the determination of the onset of oviposition and the number of eggs in an egg mass allowed an estimation of the moment the after-discharge of the CDCs had occurred. The moment of the discharge, thus calculated, was compared with the time the CWS was presented to the animal. The mean latency between the CWS and the calculated moment of discharge was in keeping with the one reported by ter Maat *et al.* (1989).

Calculations and statistical analysis

The time that elapsed between the calculated onset of the discharge of the CDCs and the onset of egg laying (the ovipository delay) differed from snail to snail. In order to allow for comparisons of the surfacing latencies at different moments of the ovipository delay among different snails, the ovipository delays were normalised. The total duration of the ovipository delay in each case was taken as 100 %, and the time which elapsed between the onset of the discharge of the CDC' s and the moment of the presentation of each surfacing stimulus was expressed as a percentage of this time interval. Thus, the horizontal axis in Fig. 1 represents a relative time scale, ranging from 0 % (moment of discharge of CDCs) to 100 % (start of oviposition). 49 paired data were obtained, consisting of a percentage of the ovipository delay and the surfacing latency at that time. These data were tested for correlation in a Spearman correlation test.

RESULTS

The behaviour of the snails was observed during 4 hours, following the application of the CWS. From the 28 animals used in this study, 19 showed egg laying within this period. The remaining animals did not show egg laying behaviour. The surfacing latencies of these animals amounted 3' 54" ± 3' 30" (mean and s.d. of 32 observations on 19 different animals). In the egg laying animals the mean latency between the CWS and the estimated time of the afterdischarge was 20' 12" ± 18' 54" (mean and s.d. of 19

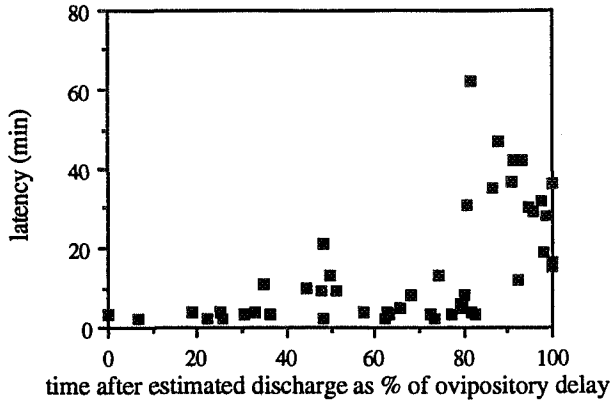


Fig. 1. Surfacing latency (min, vertical axis) plotted against the moment of stimulus application, expressed as a % of the ovipository delay (horizontal axis; 0 % corresponds with the estimated initiation of the CDC discharge, 100 % corresponds with the initiation of oviposition).

animals). The mean duration of the ovipository delay was 127' ± 28', the mean duration of oviposition 19' 24" ± 7' 42". The mean number of eggs in each egg mass was 116 ± 43.

The transient exposure of the animals to low PO_2, used in this study, and the resulting interruption of the egg laying behaviour by surfacing did not affect the number of animals that showed egg laying after the CWS, nor did it affect the number of eggs per egg mass (*cf.* ter Maat *et al.*, 1989). This indicates that the CDC discharge and the ensuing processes which ultimately result in the production of the egg mass were not noticeably affected by the presentation of the surfacing stimuli to the animals.

Fig. 1 shows the surfacing latencies at the different relative time intervals between the estimated time of discharge and the start of oviposition. The Figure shows that after the discharge the surfacing latency initially was low, and comparable to the latencies observed in the animals which did not show egg laying (see above). At approximately 80 % of the ovipository delay, however, there was a sudden increase of the surfacing latency. When a surfacing stimulus was presented to the snails within this period, the snails first entered the phase of oviposition; only after deposition of an egg mass they moved to the water surface. The Spearman correlation test revealed a significant correlation between the time of application of the surfacing stimulus (expressed as percentage of the ovipository delay) and the surfacing latency ($p < 0.001$).

DISCUSSION

The present study shows that surfacing, induced by transient exposure of the animal to low PO_2 is suppressed during part of the egg laying behaviour. The mechanisms which

underlie the suppression of this type of induced surfacing may also be partly responsible for the usually prolonged duration of submergence during egg laying. The significance of this phenomenon is likely to be related to the importance to the animal to deposit the egg mass on the substrate, prepared during the preceding, appetitive phases of the behaviour (*cf.* ter Maat *et al.*, 1989).

The extension of the period of submergence between two successive periods of aerial ventilation has been established for several bimodal breathers. In the aquatic newt *Triturus vulgaris*, a competetive interaction has been demonstrated between reproductive behaviour and aerial ventilation (Halliday and Sweatman, 1976). In the African clawed toad *Xenopus laevis*, the presentation of threatening stimuli at the water surface prolongs the duration of submergence beyond the normal duration of intervals between two successive aerial ventilations (Boutilier, 1984). With respect to control mechanisms, it has been suggested that chemosensory drive, related to respiratory gas tensions generates a basic pattern of respiratory behaviour. As soon as other activities take precedence over aerial ventilation, input related to these activities overrules this respiratory drive, resulting in deviation of the basic pattern of respiratory behaviour (Boutilier, 1988).

The results presented in Chapters 2 and 3 indicate that in *Lymnaea* too, the durations of non-ventilatory intervals and the durations of ventilatory bouts at the surface are to an important extent dependent on internal or external PO_2 or PCO_2 or internal pH. The stimulus that was used in the present experiment, viz. transient exposure of the animals to N_2, is likely to result in a decrease of the internal PO_2 (at least in the lung cavity). Since the animals usually responded to this stimulus within a short time by surfacing and subsequent ventilation, these data are consistent with a role of the PO_2 in the lung cavity or adjacent circulatory system in the control of this behaviour. However, the present data also show that *Lymnaea* is capable of ignoring these stimuli if continued submergence is required in order to finish egg laying. Thus, to the factors that affect the tendency of *Lymnaea* to orientate and move towards the water surface should be added factors that are related to egg-laying behaviour.

Demands that are set on respiratory physiology to enable prolonged submergence

The capability to remain submerged for prolonged periods of time depends on a number of parameters, including total O_2 capacity, O_2 consumption, anaerobic capacity and the PO_2 of the ambient water. There are several indications that *Lymnaea* does not meet severe respiratory problems as a result of the increased duration of submergence during egg laying. In chapter 3 we demonstrated that in spite of the decrease of haemolymph PO_2 which occurs during submergence, heart rate is unaffected and there are no signs of anaerobic metabolism. Indeed, in normoxic water, *Lymnaea* can survive periods of forced submergence indefinitely (Wijsman, pers. comm.). In anoxic water, its tolerance to forced submergence is approximately 40 hours (Wijsman *et al.*, 1985). Thus, its

tolerance to submergence amply allows in both cases for submergence for the period during egg laying. Moreover, egg laying is not likely to occur in O_2 poor water since the O_2 content of the ambient water has been shown to be one of the prerequisites for the occurrence of egg laying (ter Maat *et al.*, 1983).

Possible neural mechanisms underlying suppression of surfacing

The overt egg laying behaviour in *Lymnaea* is subdivided into a quiescent phase (duration typically 60 min), starting approximately 15 min after the CWS, followed by a turning phase (also 60 min), and an ovipository phase. This sequence of events is not or only rarely interrupted by surfacing. The present study shows that suppression of induced surfacing behaviour occurs in the interval between 80 % of the ovipository delay and the end of oviposition. 80 % of the ovipository delay corresponds to a period of approximately 20 min before the initiation of oviposition. During the preceding part of the ovipository delay, surfacing latencies are approximately equal to those observed in non-egg laying animals. This shows that, although during this part of the ovipository delay locomotory activity is normally low, surfacing and the accompanying locomotion can still be induced.

With respect to neural mechanisms, indications have been obtained that the timing of the different phases of egg laying behaviour arises from the interaction between neuroactive peptides, released by the CDCs, and feedback originating from the covert behaviours in the reproductive tract (ter Maat *et al.*, 1989). Clearly, suppression of surfacing does not coincide with the occurrence of the after discharge in the CDCs. As the period of suppression of surfacing is not temporally related to the occurrence of the discharge, it seems unlikely that suppression of surfacing is due to the direct action of neuroactive peptides released during the CDC discharge. The period during which suppression of surfacing occurs (before the actual deposition of the egg mass) is part of the turning phase. It has been suggested that the transport of eggs in the reproductive tract is instrumental in determining the duration of the turning phase (ter Maat *et al.*, 1989). Hence, it is conceivable that suppression of surfacing too, is mediated by sensory structures sensing the transport of the egg mass in the reproductive tract.

REFERENCES

Adamo S. A. and Chase R. (1991a). The interaction of courtship, feeding and locomotion in the behavioural hierarchy of the snail *Helix aspersa*. Behav. Neural Biol. **55** : 1 - 18.

Adamo S. A. and Chase R. (1991b). "Central arousal" and sexual responsiveness in the snail *Helix aspersa*. Behav. Neural Biol. **55**: 194 - 213.

Boutilier, R. G. (1984). Characterization of the intermittent breathing pattern in *Xenopus laevis*. J. Exp. Biol. 110, 291 - 309.

Boutilier, R. G. (1988). Control of breathing in bimodal breathers: Amphibia. Can. J. Zool. **66**: 6 - 19.

Geraerts, W. P. M., ter Maat, A. and Vreugdenhil, E. (1988). The peptidergic neuroendocrine control of egg laying behavior in *Aplysia* and *Lymnaea.. In* Endocrinology of Selected Invertebrate Types, pp. 141 - 231. Alan R. Liss, New York.

Halliday, T. R. and Sweatman, H. P. A. (1976). To breathe or not to breathe: the newt's problem. Anim. Behav. **24**: 551 - 561.

ter Maat, A., Lodder, J. C. and Wilbrink, M. (1983). Induction of egg-laying in the pond snail *Lymnaea stagnalis* by environmental stimulation of the release of ovulation hormone from the Caudo-Dorsal Cells. Int. J. Invertebr. Reprod. **6**: 239 - 247.

ter Maat, A., Pieneman, A. W., Goldschmeding, J. T., Smelik, W. F. E. and Ferguson, G. P. (1989). Spontaneous and induced egg laying behavior of the pond snail, *Lymnaea stagnalis*. J. Comp. Physiol. **164**: 673 - 683.

van der Steen, W. J., van den Hoven, N. P. and Jager, J. C. (1969). A method for breeding and studying freshwater snails under continuous water change, with some remarks on growth and reproduction in *Lymnaea stagnalis* (L.). Neth. J. Zool. **19**: 131 - 139.

Wijsman, T. C. M., van der Lugt, H. C. and Hoogland, H. P. (1985). Anaerobic metabolism in the freshwater snail *Lymnaea stagnalis*: Haemolymph as a reservoir of D-lactate and succinate. Comp. Biochem. Physiol. **81B**: 889 - 895.

Ziv I., Lustig C., Ben - Zion M. and Susswein A. J. (1991a). Daily variation of multiple behaviours in *Aplysia fasciata*: Integration of feeding, reproduction and locomotion. Behav. Neural Biol. **55**: 86 - 117.

Ziv I., Markovich, S., Lustig C. and Susswein A. J. (1991b). Effects of food and mates on time budget in *Aplysia fasciata*: Integration of feeding, reproduction and locomotion. Behav. Neural Biol. **55**: 68 - 85.

CHAPTER 5

INTERACTION BETWEEN RESPIRATORY AND FEEDING BEHAVIOUR IN THE FRESHWATER PULMONATE SNAIL *LYMNAEA STAGNALISS*

SUMMARY

We monitored the respiratory and feeding behaviour of fed and starved snails in normoxic and anoxic conditions in jars with lettuce placed on the bottom. At anoxia, snails show an increased tendency to move towards the surface of the water in order to carry out aerial ventilation. Hence starved snails will have to choose in such conditions whether to surface and breathe, or to remain submerged and feed. Starvation increased the amount of time the snails spent feeding; it did not significantly affect the amount of time spent breathing. Anoxia increased the amount of time the snails spent breathing and it decreased the amount of time the snails spent feeding. We conclude that in *Lymnaea* the feeding condition may considerably affect the normal pattern of surfacing, aerial ventilation and submergence. The feeding condition can be considered as yet another factor which - besides factors which are related to changes in PO_2 and PCO_2 - affects the tendency of the animal to orientate and move to the water surface.

INTRODUCTION

In the preceding Chapters, we studied a number of aspects of bimodal breathing in *Lymnaea stagnalis*. On the basis of these results, we postulated that in *Lymnaea* the tendency to orientate and move towards the water surface is affected by the PO_2 and PCO_2 of the ambient water; at the surface, the tendency to open and close the pneumostome is to a large extent affected by the PO_2 and PCO_2 of the inspired air. Because of the requirement to surface in order to carry out aerial ventilation, bimodal breathing is much more likely to interfere with other types of behaviour as compared to unimodal breathing. In order to minimize this interference, some flexibility may be required in the responses of *Lymnaea* to the primary factors that control its ventilatory behaviour. In the preceding Chapter, it was shown that the response of *Lymnaea* to stimuli that normally induce surfacing and subsequent aerial ventilation may be suppressed during part of the egg laying behaviour. In the present paper, we studied interactions between aerial ventilation and feeding. In its natural environment, *Lymnaea*

mainly finds an important part of its food on the bottom of the water (Scheerboom and Van Elk, 1978). This implies that the necessity to remain in the proximity of the water surface in order to breathe, is conflicting with the necessity to move to lower depths of the water in order to feed. Such conflicts, and the changes in orientation in relation to food location and the necessity to breathe have been reported to occur in the fresh water snail *Planorbis* (Deliagina and Orlovsky, 1990). Effects of food location and oxygen content of the water on feeding, ventilation and migratory patterns of *Lymnaea stagnalis* have indeed been reported by Jager *et al.* (1979). In order to study these interactions between ventilatory and feeding behaviour further, we subjected snails to deprivation of food, deprivation of O_2 or both and determined the distribution of the time the animals spent under the various conditions to either feeding or respiration. The results clearly show that after five days of starvation, *Lymnaea* can remain submerged feeding for periods that are much longer than those observed in snails that are fed normally; this holds for both normoxic and anoxic conditions. This suggests that *Lymnaea* can respond in a flexible way to primary factors controlling ventilatory behaviour.

MATERIALS AND METHODS

Adult specimens of *Lymnaea stagnalis* (L.), shell length about 30 mm were used. They were bred under laboratory conditions at a 12-12 h light-dark regimen and were fed lettuce every other day (van der Steen, van den Hoven and Jager, 1969). Five days prior to the experiment, snails were transferred and kept individually in jars and either starved or fed lettuce ad libitum. For the behavioural observations, snails were transferred to jars, containing 500 ml of tap water (20 °C), with lettuce placed on the bottom of the jar. The water was previously equilibrated with pressurized air (PO_2 150 Torr, normoxic) or N_2 (PO_2 < 15 Torr, anoxic). When snails were studied in anoxic conditions, a continuous flow of N_2 over the space above the water maintained low PO_2 in water and gas during the experiment. Snails were allowed to acclimatize for 5 min before the observations were started. From the data, presented in Chapter 3, it can be inferred that within this period, haemolymph PO_2 had decreased below the level, measured in snails that were kept in normoxic conditions. Feeding and respiratory behaviour were observed visually. The amount of time the snails spent submerged with or without feeding and surfacing with or without breathing were timed with a stopwatch and expressed as the percentage of the total time the animal was observed (1 hour). Four experimental groups were studied, each consisting of 8 different snails. Snails were either starved (5 days food deprivation) or fed (fed lettuce ad libitum), and studied at normoxia (PO_2 of water and air approximately 150 Torr) or anoxia (PO_2 of water and air < 15 Torr). PO_2 was measured with a polarographic O_2 electrode (Instech, model 125/05) connected to a single channel O_2

amplifier (Instech, model 102 B/230). Statistical tests were performed in a two way analysis of variance.

RESULTS

The behaviour of snails that had been either fed or starved prior to the experiment was observed while they were kept in either normoxic or anoxic conditions. We monitored whether the animals were under water, either feeding or not, or at the surface, either breathing or not. Figure 1 A shows how snails distributed time among the four different categories of behaviour in the different conditions. The four levels in the figure represent (from top to bottom): breathing, at the surface (not breathing), under water (not feeding), and feeding (see Figure 1 B). In Figure 1 A a representative example is shown for each experimental group. The average percentages of time spent to each type of behaviour and the results of the two-way ANOVA, testing the significance of the major effects (starvation and anoxia) and their interactions on time budgetting are shown in Table 1.

The effect of the feeding condition depended on the respiratory condition of the animals. As expected, starved snails spent greater amounts of time feeding. This effect was much stronger under normoxia than under anoxia. Apparently, the increased tendency in starved snails to feed is reduced as the result of anoxic conditions. This effect also appears from the traces in Figure 1. At normoxia, the effect of starvation is quite dramatic. Starved animals spent the major part of the time submerged; feeding was only rarely interrupted through excursions to the water surface to breathe. This resulted in periods of submergence lasting several tens of minutes. Under anoxia, the feeding of starved snails was interrupted more regularly by surfacing. In spite of this, during anoxia too, starved snails spent relatively longer periods of time feeding as compared to fed snails. At anoxia, the bouts of ventilatory movements lasted longer as compared to those observed at normoxia.

Anoxia resulted in an increase of the amount of time the snails spent breathing. This was true for fed as well as starved snails. This effect can be readily seen in Figure 1. In fed and in starved snails, anoxia resulted in a decrease of the period between successive bouts of ventilation. Moreover, the durations of the ventilatory bouts were longer as compared to normoxia.

The effect of the feeding condition on the fraction of time the animals spent submerged while not feeding, was dependent on the respiratory condition of the animals. The decrease was stronger during normoxia than during anoxia. The traces in Figure 1 show that at normoxia, fed snails spent most of the time submerged; for the major part of this time, they were not feeding. Starved snails also spent most of the time submerged at normoxia, but for the major part feeding. A similar, but less strong effect occurred at anoxia.

Finally, anoxia caused a significant increase of the fraction of time the animals spent at the surface, while not breathing. In contrast, starvation caused a significant decrease of this parameter. These effects were independent of each other. In Figure it can be seen that anoxia caused an increase of the number and duration of ventilatory bouts. These bouts included periods during which the pneumostome was closed. During starvation, the animals remained submerged for prolonged periods of time; excursions to the water surface occurred less frequently. As a consequence of this, the fraction of time the animals spent at the water surface with pneumostome closed was decreased.

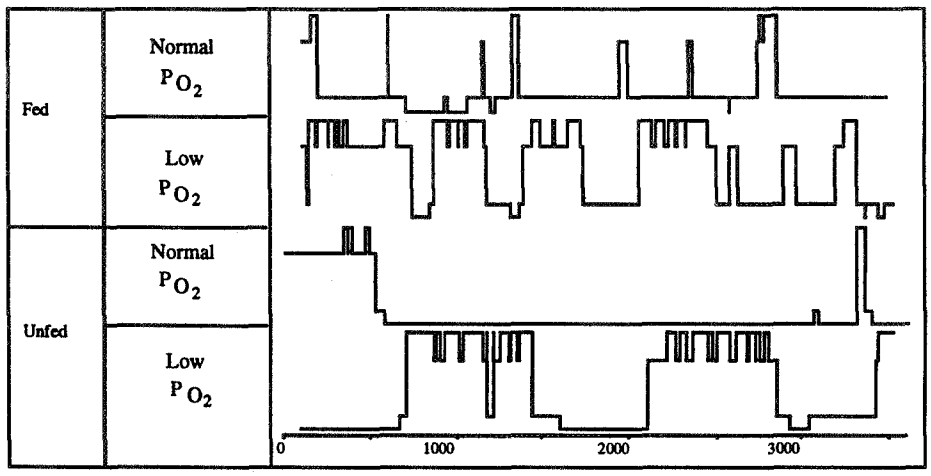


Figure 1 A. Schematic representation of the periods of time *Lymnaea* spent (1) submerged and feeding, (2) submerged, (3) surfacing and (4) surfacing and breathing. Snails were either fed or starved prior to the experiment, and subjected to normal or low PO_2 in the ambient water and inspired air during the experiment.

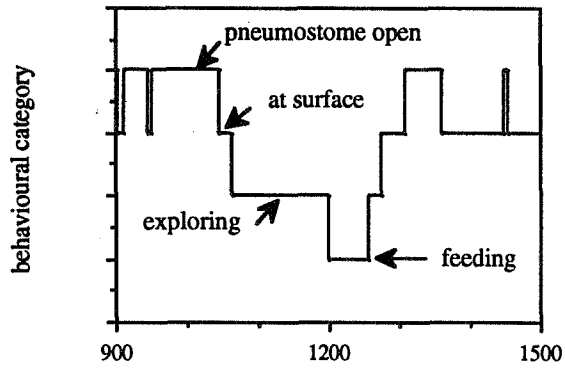


Figure 1 B. Explanation of the correspondence between the type of behaviour displayed by the animal and the level of the traces in the schematic representation in Figure 1 A.

Table 1. Relative amounts of time spent feeding, breathing, submerged without feeding and surfacing without breathing as expressed as the percentage of total observation time (1 hour) in fed- and starved snails at normoxia or anoxia. Mean and standard deviations of observations on 8 different snails per group. Estimated p-values of the main effects of the feeding- and the respiratory condition of the animals and their interactions on the foregoing parameters (two-way analysis of variance).

	Fed	Starved	
% feeding	19.8 ± 10.5	87.9 ± 7.3	normoxia
	6.7 ± 9.7	33.5 ± 23.9	anoxia
% submerged (not feeding)	60.1 ± 17.7	6.3 ± 4.0	normoxia
	36.0 ± 10.8	14.6 ± 12.1	anoxia
% breathing	10.7 ± 6.4	0.9 ± 1.3	normoxia
	35.3 ± 10.4	41.8 ± 21.3	anoxia
% surfacing (PN closed)	9.4 ± 8.1	5.1 ± 5.1	normoxia
	22.1 ± 10.4	10.1 ± 5.1	anoxia

	% feeding	% submerged (not feeding)	% breathing	% surfacing (not breathing)
feeding condition	<0.001	<0.001	>0.05	0.005
respiratory condition	<0.001	>0.05	<0.001	0.003
interaction	<0.001	<0.001	>0.05	>0.05

DISCUSSION

The results clearly show that in *Lymnaea* respiratory behaviour and feeding behaviour can interfere with each other. Since in its natural environment *Lymnaea* mainly feeds on the bottom, this interference is likely to occur under natural conditions too. This will present especially problems at low PO_2 of the ambient water, because of the greater reliance of *Lymnaea* on aerial ventilation under these conditions. Obviously, the necessity to surface in order to breathe air prevents the animal from remaining submerged and feeding. From the data in Chapter 2, we concluded that at low PO_2 of the ambient water, the tendency of the animal to orientate and move towards the water surface is increased. At the surface, low PO_2 of the inspired air increases the tendency of the animal to open and close the pneumostome, thereby preventing it from returning to the lower depths of the water again. In the present experiments, the result of anoxia is especially clear in starved snails: whereas during normoxia the animals spend most of the time submerged feeding, at anoxia the animals spend relatively long periods of time at the surface, carrying out many, short lasting pneumostome movements that are characteristic of anoxia. Periods of submergence are short as compared to normoxia.

Whereas aerial ventilation may be considered to interfere with feeding, especially during low O_2 availability, the reverse also holds: feeding, especially after a period of starvation, may interfere with the basic pattern of aerial ventilation. When fed snails were studied under normoxic conditions, a pattern of respiratory behaviour was observed that resembles the pattern that was described in Chapter 2. The animals spend the major amount of time under water; if the animals move to the surface, surfacing periods are usually short, accompanied by one or two ventilatory movements of the pneumostome. On average, approximately 6 respiratory bouts were carried out during one hour, resulting in mean intervals between two successive bouts of approximately 10 minutes. These data are consistent with the results of Chapter 2. Although starvation did not significantly affect the amount of time the animals spent breathing, the traces in Figure 1 clearly show that generally, starved snails remained feeding under water during longer periods than fed snails. This is especially clear under normoxia, where snails remained submerged feeding for the major part of the time; feeding was only rarely or not at al interrupted by excursions to the water surface for breathing. Clearly, *Lymnaea* is capable of deviating considerably from the pattern of surfacing and submergence that is normally observed in fed snails during normoxic conditions. This suggests that the factors that are normally sufficient to initiate surfacing and subsequent aerial ventilation, are overruled by the motivation of the animal to remain submerged and continue feeding.

From the results, presented in Chapters 2, we concluded that internal and external PO_2 and PCO_2 are important factors that control ventilatory behaviour in *Lymnaea*. We also concluded that *Lymnaea* does not respond to these factors in a rigid way; also at low

ambient PO_2 and high ambient PCO_2, *Lymnaea* does terminate bouts of ventilation at the surface and returns to the lower depths of the water again. From the data in Chapter 3 we concluded that *Lymnaea* tolerates changes in O_2 availability relatively well; its bimodal mode of breathing entails relatively large fluctuations in haemolymph PO_2. If haemolymph PO_2 decreases below a certain level, *Lymnaea* has the capacity to switch to anaerobiosis. The significance of the flexibility of its response and the flexibility in its O_2 requirements may be related to minimising the risk of interference with other types of behaviour. This flexibility allows the animal to remain submerged for prolonged periods of time in order to feed, especially after periods of starvation, in O_2 rich as well as O_2 poor water.

Our data also show that probably no rigid prevalence exists for feeding or breathing in *Lymnaea*. The animals did not consistently display preference to one of these behaviours at the expense of the other. Rather, when forced to choose, the behaviour should probably be considered as a flexible outcome of competing drives, similar to what has been described for *Navanax inermis* (Leonard and Lukowiak, 1984). The relative contribution of each of the two drives is likely to be related to the extent of deprivation. We employed a period of 1 hour anoxia because changes in haemolymph PO_2 and anaerobiosis are known to occur within this period; the maximal period of complete O_2 deprivation tolerated by *Lymnaea* is *ca.* 40 h (Wijsman, van der Lugt and Hoogland, 1985). The period of starvation was chosen arbitrarily; *Lymnaea* survives starvation for periods of several weeks (*cf.* Bohlken *et al.*, 1986).

Our data indicate that among the factors which control initiation and termination of ventilatory behaviour in *Lymnaea*, the respiratory and feeding condition of the animal can show competitive interactions. With respect to control mechanisms of diving behaviour, it has been suggested that in amphibian bimodal breathers, a basic pattern of respiratory behaviour is generated, resulting primarily from O_2- and CO_2 chemosensory input. This basic pattern may be overruled, when other types of activity take precedence over respiratory behaviour (Boutilier, 1988). Our data suggest that the neural network, involved in respiratory control, is affected by neural or hormonal factors, related to the feeding condition of the animal. The neural network, involved in control of feeding and the possible mechanisms, underlying satiation in *Lymnaea* have been described (Benjamin, Elliott and Ferguson, 1985; Tuerseley and Mac Crohan, 1987). In addition, data are available on the processing of O_2 chemosensory information in the nervous system, on the neuronal network, involved in control of respiration of *Lymnaea* (Chapters 6 and 7) and on the neuronal structures, involved in gravity orientation (Janse, 1982 and Janse *et al.*, 1988). Moreover, the changes that occur in the composition of the haemolymph during anoxia are known (Wijsman *et al.* 1985). Lukowiak and Freedman (1983) have shown that effects that result from differences in the behavioural state of intact animals, can persist in reduced preparations. Hence, it seems feasible to study the

physiological mechanisms, underlying the competitive interactions between feeding and respiratory drives.

REFERENCES

Benjamin, P. R., Elliott, C. J. H. & Ferguson, G. P. (1985). Neural network analysis in the snail brain. *In* A. I. Selverston (Ed.), *Model Neural Networks and Behavior* (pp. 87 - 108). New York: Plenum Press.

Bohlken, S., Joosse, J., van Elk, R. and Geraerts, W. P. M. (1986). Interaction of photoperiod and nutritive state in female reproduction of *Lymnaea stagnalis*. Int. J. Invertebr. Reprod. Dev. **10**: 151 - 157.

Boutilier, R. G. (1988). Control of arrhythmic breathing in bimodal breathers: Amphibia. Can. J. Zool. **66**: 6 - 19.

Deliagina , T. G. and Orlovsky, G. N. (1990). Control of locomotion in the fresh water snail *Planorbis corneus*. I. Locomotory repertoire of the snail. J. Exp. Biol. **152**: 389 - 404.

Jager J. C., N. Middelburg - Frielink, J. W. Mooij - Vogelaar and J. W. van der Steen. (1979). Effects of oxygen and food location on behaviour in the freshwater snail *Lymnaea stagnalis* (L.). Proc. of the KNAW **C82**: 177 - 180.

Janse, C. (1982). Sensory systems involved in gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **145 A**: 311 - 319.

Janse, C., van der Wilt, G. J., van der Roest, M. and Pieneman, A.W. (1988). Intracellularly recorded responses to tilt and efferent input of statocyst sensory cells in the pulmonate snail *Lymnaea stagnalis*. Comp. Biochem. Physiol. **90A**: 269 - 278.

Leonard, J. L. & Lukowiak, K. (1984). An ethogram of the sea slug, *Navanax inermis* (Gastropoda, Opistobranchia). Zeitung für Tierpsychologie **65**: 327 - 345.

Lukowiak, K. & Freedman, L. (1983). The gill withdrawal reflex is suppressed in sexually active *Aplysia*. Canadian Journal of Physiology and Pharmacology **61**, 743 - 748.

Scheerboom, J. E. M. and Van Elk, R. (1978). Field observations on the seasonal variations in the natural diet and the haemolymph-glucose concentration of the pond snail *Lymnaea stagnalis* (L.). Proc. Kon. Ned. Akad. Wet., Series C, **81**: 365 - 376.

van der Steen, W. J., van den Hoven, N. P. and Jager, J. C. (1969). A method for breeding and studying freshwater snails under continuous water change, with some remarks on growth and reproduction in *Lymnaea stagnalis* (L.). Netherlands Journal of Zoology **19**: 131 - 139.

Tuersley, M. D. & McCrohan, C. R. (1987). Food arousal in the pond snail, *Lymnaea stagnalis*. Behavioral and Neural Biology **48**: 222 - 236.

Wijsman, T. C. M., van der Lugt, H. C. & Hoogland, H. P. (1985). Anaerobic metabolism in the freshwater snail *Lymnaea stagnalis*: haemolymph as a reservoir of D - lactate and succinate. Comparative Biochemistry and Physiology **81B**: 889 - 895.

CHAPTER 6

CENTRAL AND PERIPHERAL NEURONES INVOLVED IN OXYGEN RECEPTION IN THE PULMONATE SNAIL *LYMNAEA STAGNALIS* (MOLLUSCA, GASTROPODA)

SUMMARY

1. Upon lowering P_{O_2} over the mantle area, peripheral nerves increased their activity.

2. Intracellular recordings showed that neurones in the CNS of *Lymnaea stagnalis* are sensitive to changes in the P_{O_2} over the mantle area. Some neurones responded with hyperpolarization and decrease of the firing frequency, others with depolarization and increase of the firing frequency.

3. The statocyst sensory cells receive efferent input from oxygen-sensitive receptors in the mantle-lung area.

INTRODUCTION

The previous chapters showed that the tendency of *Lymnaea* to orientate and move towards the water surface is increased at low PO_2 of the ambient water. At the water surface, the tendency to open and close the pneumostome is increased at low PO_2 of the inspired air. We concluded that O_2 receptors, located in the skin, the internal lining of the lung cavity or the adjacent circulatory system may be involved in mediating these responses.

On the basis of behavioural studies, the presence of oxygen-sensitive receptors in the lung cavity and in the skin of *Lymnaea*, affecting orientation in the gravitational field had already been postulated by Janse (1981). The sensory structures that are involved in control of geotaxis in *Lymnaea* are the statocysts (Geuze, 1968; Janse, 1981; 1982). These are sphere shaped structures on the dorsal part of the pedal ganglia of the central nervous system (CNS), containing about 13 sensory cells (Geuze, 1968; McKee and Wiederhold, 1974; Wolff, 1975; Janse 1983). To account for the effects of ambient PO_2 changes on geotactic behaviour, Janse (1981) suggested that the statocyst sensory cells receive efferent input from O_2 chemosensitive structures, located in the lung cavity or the skin. In pulmonate snails indications for such efferent innervation have been described by Wolff (1970; 1975).

The experiments described in this chapter were carried out in order to examine whether electrophysiological data could be obtained for the presence of O_2 chemosensitivity in the lung - mantle area of *Lymnaea* and the processing of this information in the central nervous system. For this purpose, the electrical activity of nerves and of central neurones, including the statocyst cells, were recorded in semi-intact preparations while varying the P_{O_2} in the lung - mantle area. The results show that exposure of the lung - mantle area to low PO_2 results in an increased activity of a limited number of elements in two nerves innervating this area. From the central neurones that were recorded from in this study, a number responded specifically to low PO_2 in the lung - mantle area, either by increased or by decreased activity. Among these, some but not all of the statocyst cells responded with a decreased acitivity to low PO_2 in the lung - mantle area. These findings are considered to substantiate the postulated presence of oxygen chemosensitivity in those parts of the preparation that were exposed to air with normal and low PO_2 and the hypothesis of efferent innervation of the statocyst cells.

MATERIALS AND METHODS

Laboratory-bred snails with a shell length of approximately 30 mm were used. Two types of preparations were made:

(1) Preparations used to record from peripheral nerves. These preparations consisted of the mantle and the peripheral nerves. The lung cavity was exposed by a transverse cut in the lung roof. All visceral organs were removed except those situated against the lung wall (kidney, heart and pericard, oothecal gland and bursa copulatrix). The tissue around the pneumostome was left intact. The nerves were cut close to the CNS. Preparations were exposed to N_2 gas or air by placing the recording basin in a large compartment in which the air could be replaced (Fig. 1). During exposures to gas the Ringer's solution in the recording basis was partly removed such that the mantle area was exposed to gas while the nerves remained covered with Ringer's solution. P_{O_2} measurements were made during the experiment at the outflow of the gas compartment. This means that a latency of several seconds existed between the measured and the actual P_{O_2} over the recording basin.

(2) Preparations used to record from central neurones. They consisted of the mantle (including the lung) connected to the CNS via the visceral and parietal nerves (Fig. 2). Here too, the lung caivity was exposed by a transverse cut in the lung roof. The preparation was pinned down in a basin consisting of two separate compartments in which mantle and CNS were positioned, respectively. Each compartment had a volume of about 4 ml. The separation between the compartments was air- and water-tight. In the mantle compartment, the nerves were covered with grease. The Ringer solution in the mantle compartment could be removed separately and the compartment could be filled

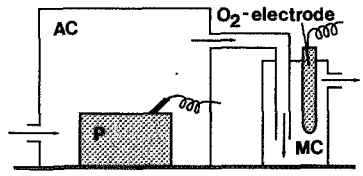


Fig. 1. Experimental set-up for the recordings of activity of peripheral nerves during exposures of the mantle to different P_{O_2}. AC, Compartment in which air could be replaced; MC, Compartment in which the P_{O_2} was measured during the recordings; P, Recording chamber in which the preparation was pinned down; E, Suction electrode. Arrows indicate the flow of gas.

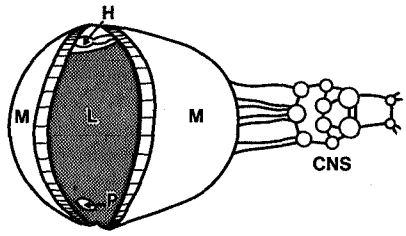


Fig. 2. Diagram of the mantle-CNS preparation: M, mantle; L, lung cavity; H, heart and pericard; P, pneumostome; CNS, central nervous system. See also text.

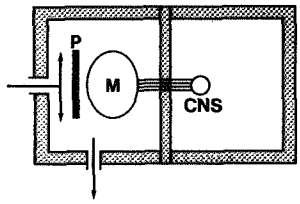


Fig. 3. Diagram of the set-up used for the recordings from central neurones. Arrows indicate gas flow. P, Perspex plate to prevent direct gas flow over the preparation.

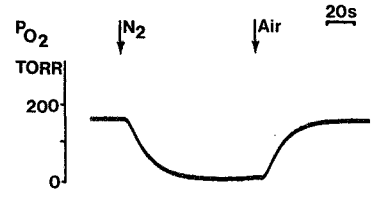


Fig. 4. Recording of P_{O_2} in the compartment of Fig. 2 (containing the mantle) during replacements of gas. Arrows indicate the onset of gas flow.

with air or with N_2 gas while it was covered with a glass plate. A vertically placed perspex plate prevented direct gas flow over the mantle. Gas could leave the compartment through an outlet (Fig. 3). Separate P_{O_2} measurements in the mantle compartment (Fig. 4) showed that it took about 30-40 sec to replace the air completely with N_2 gas.

In the experiments on both types of preparations three types of gas exposures were used: (1) Air; in this case only the Ringer solution was removed: (2) Flowing air: pressurized air was blown into the compartment containing the preparations; (3) N_2 gas: pressurized N_2 gas was blown into the compartment. The velocity of gas flow was controlled by the gas pressure at the outlet of the gas containers. Gas pressure was controlled by a valve. During different P_{O_2} measurements in the mantle compartment (second type of preparation) only minor differences occurred.

Intracellular recordings were made with glass micropipettes with a resistance of 10-50 MΩ. Extracellular recordings were made with glass-suction electrodes. Conventional electrophysiological techniques were used for recording, storing and displaying signals. A window discriminator (WPI) was used to select extracellular signals. A digital counter and an interval to frequency converter were used to count action potentials and measure their instantaneous frequency, respectively. Tactile stimuli were delivered with a hand-worked glass probe with a rounded tip (diameter: 0.5 mm). P_{O_2} measurements were made with a WTW oxy 42 oxygen electrode or with a P_{O_2}-electrode made in the laboratory. The experiments were performed at room temperature with a Ringer's solution of the following composition: NaCl, 30 mM; KCl, 1.5 mM; $MgCl_2$, 2 mM; $CaCl_2$, 4 mM; NaH_2PO_4, 0.25 mM; $NaHCO_3$, 18 mM.

RESULTS

In the following experiments preparations were stimulated by exposing the periphery to air or N_2 gas. Mantle preparations were used because the mantle area consists of an area of external skin which is normally exposed to water, as well as the lung cavity which is normally exposed to air; in both structures oxygen-sensitive receptors are probably present (Janse, 1981).

The mantle is innervated by the nerves of the visceral and parietal ganglia (Janse, 1974). Therefore, in the present study attention was primarily focused on the involvement of neurones in these ganglia in O_2 reception. The nomenclature of Wendelaar Bonga (1970), Benjamin and Winlow (1981) and Swindale and Benjamin (1976) was used for the different types of cells. Figure 5 gives a diagram of the CNS with the location of the neurones used in the present study.The responses described below for the Right Pedal Dorsal 1 neurone ($RPeD_1$) and the Visceral Dorsal Neurones 2 and 3 (VD_2/VD_3) and the peripheral afferents were each recorded in at least four to five preparations; the responses

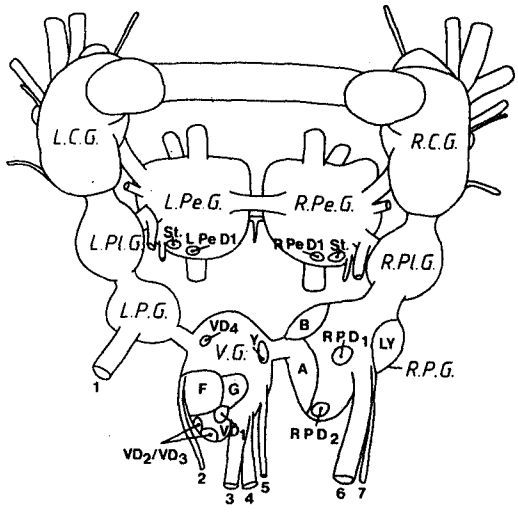


Fig. 5. Dorsal view of the CNS of *Lymnaea stagnalis* (after Benjamin and Winlow, 1981 and Slade *et al.*, 1981). LCG and RCG, left and right cerebral ganglion; LPeG and RPeG, Left and right pedal ganglion; LPlG and RPlG, left and right pleural ganglion; LPG and RPG, Left and right parietal ganglion; VG, Visceral ganglion. (1) n. pallialis sinister; (2) n. cutaneous pallialis; (3) n. intestinalis; (4) n. analis; (5) n. genitalis; (6) n. pallialis dexter internus; (7) n. pallialis dexter externus. $LPeD_1$ and $RPeD_1$, left and right pedal dorsal cells; VD_1-VD_4, visceral and dorsal cells; RPD_1 and RPD_2, right parietal dorsal cells. A, B, F, G, Y, LY: A, B, F, G, yellow, and light yellow cells, respectively, St, statocyst.

in the Visceral Dorsal 1 neurone (VD_1) and the Right Parietal dorsal 2 neurone (RPD_2) and in the G, B and F cells in eight or more preparations.

Afferent signals in the nerves during exposure of the mantle area to P_{O_2} changes

In these experiments the first type of preparation was used, consisting of the mantle with opened lung cavity and with peripheral nerves cut close to the CNS. During these experiments the mantle part was successively exposed to air, to N_2 gas and then flowing air. Activity was recorded in one of the pallial nerves.

Figure 6 shows the recording of the activity of the nervus analis during exposures of the mantle area to different P_{O_2}. Simultaneously, the instantaneous frequency of the action potentials with a particular amplitude was measured. The figure shows that during exposure to low P_{O_2} there is an increased activity in the nerve. Analysis of the neural activities revealed that especially neural elements with action potentials of a medium amplitude were involved in the response. Figure 7 shows that there is an increase in the mean frequency of these elements during the second minute of exposure of the mantle area to N_2 gas. In some other preparations, the increase in the firing rate was noticeable in the first minute. Similar results were obtained with recordings of the nervus pallialis dexter externus (not shown). Thus, these experiments indicate that a selective number of neural elements are activated during exposure of the mantle area to air with low P_{O_2}.

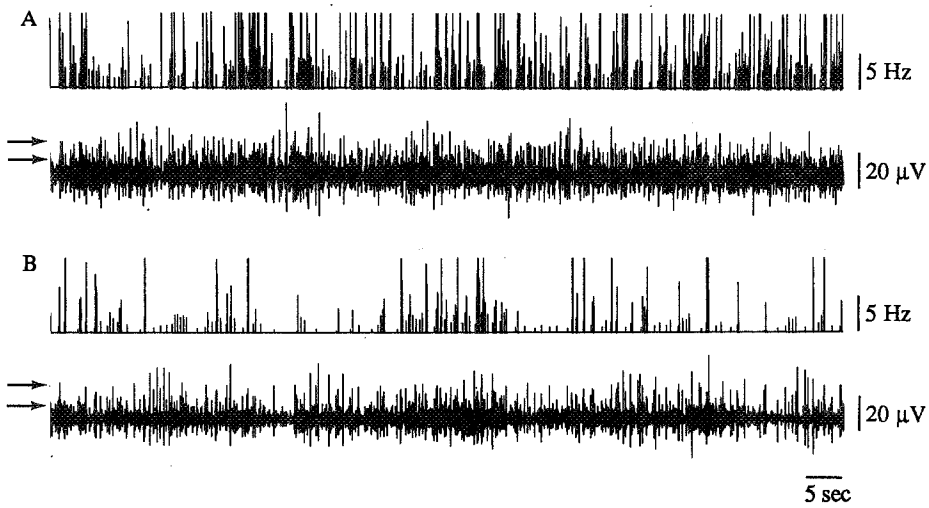


Fig. 6. Activity of the N. analis during exposures of the mantle area to air with low P_{O_2} (A, 8-2.5%) and air with high P_{O_2} (B, 14-20%). Upper traces indicate the instantaneous frequency of the action potentials which were selected by the window (upper and lower levels are indicated by the arrows) in the lower recordings. The recordings are from the experiment of Fig. 7.

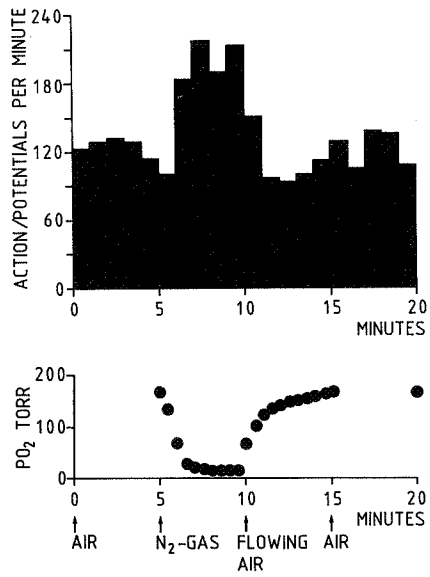


Fig. 7. Mean frequency of the action potentials selected by the window in Fig. 6 during changes of the P_{O_2} (lower graph) induced by the exposures of the preparation to air (Air), N_2 gas and flowing air. Countings were made from the recordings of the experiment illustrated in Fig. 6.

Responses of central neurones to exposure of the mantle to air or N_2 gas

Neurones on the dorsal and the ventral surfaces of the visceral and parietal ganglia were impaled. Only those neurones, present on the dorsal surface of the visceral and the right

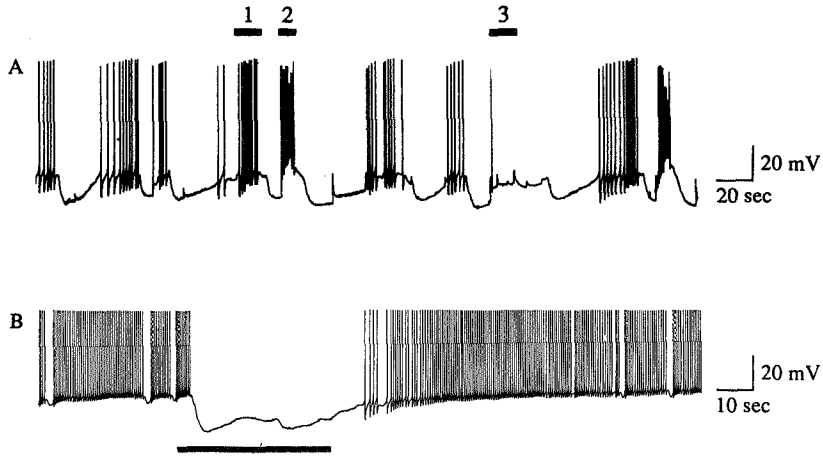


Fig. 8. A. Responses of VD_1 to mechanical stimulations (horizontal bars) of the pneumostome area (1); mantle edge (2), and the wall of the lung cavity (3). B. Recording of the activity of VD_1 during exposure of the mantle area to air and N_2 gas (bar).

parietal ganglia responded to changes in the P_{O_2}. In addition to the neurones in the visceral and parietal ganglia, recordings were also made from RPeD1, of the right pedal ganglion, since this neurone has branches in the parietal and right visceral ganglion and receives inputs in common with neurones in these ganglia (Benjamin and Winlow, 1981; Haydon and Winlow, 1981). Neuronal responses to P_{O_2} changes over the mantle area were compared to mechanical stimulations of different parts of the mantle and the lung cavity. Possible effects of direct mechanical stimulation by gas flowing over the skin were examined by comparing the neuronal responses to flowing N_2 gas, to those obtained with similar exposures to flowing air. A neurone was considered to respond to P_{O_2} changes over the mantle when its activity during flowing N_2 gas exposure differed from that during exposure to flowing air.

Responses of identified neurones

(a) VD_1 and RPD_2

VD_1 and RPD_2 are giant neurones (diameter: ± 150 µm) located in the visceral and right parietal ganglion, respectively. They are electrotonically coupled and show positive immuno reaction to anti-ACTH; their axonal branches extend through the entire CNS (Boer *et al.*, 1979; Benjamin and Winlow, 1981; Kerkhoven *et al.*, 1991). These neurones produce cardioactive peptides that show homology with the peptides of *Aplysia* neurone R15 (Bogerd, 1992). In isolated CNS preparations these neurones have a regular firing pattern (Benjamin and Winlow, 1981).

In the semi-intact preparations, used in the present study, these neurones fired burst of action potentials which were interrupted by periods of inhibition lasting up to 30 sec

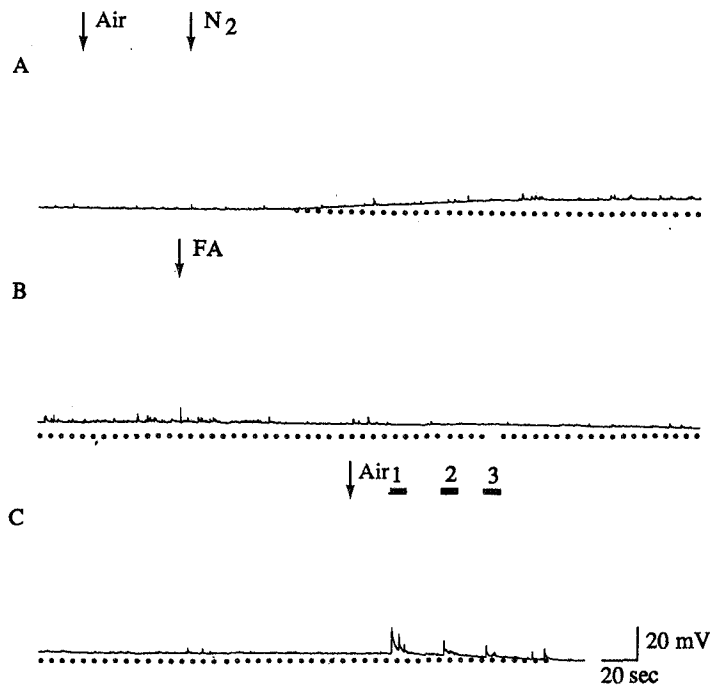


Fig. 9. A,B,C. Continuous recordings of VD_2/VD_3 during exposures of the mantle area to air (Air), N_2 gas and flowing air (FA) and during mechanical stimulation of the mantle edge (1); pneumostome (2), and wall of the lung cavity (3). The dotted line indicates the resting level of the membrane potential.

(Fig. 8B). Our experiments show that these neurones received strong excitatory input upon mechanical stimulation of different parts of the mantle and the area near the pneumostome, and weak excitatory input from the internal lining of the lung cavity (Fig. 8A). Exposure of the periphery to N_2 gas produced, after a latency of about 10 sec, a strong hyperpolarization of approximately 10 mV and a decrease in the number of action potentials. Upon exposure of the periphery to flowing air these neurones resumed their normal activity (Fig. 8B).

(b) VD_2 and VD_3

These cells are situated in the visceral ganglion near VD1. They are electrophysiologically, as well as morphologically indistinguishable from each other (Benjamin and Winlow, 1981). In our preparations these cells were normally silent, which agrees with the situation in the isolated CNS (Benjamin and Winlow, 1981). Upon mechanical stimulation of the mantle, pneumostome and lung wall these cells responded with excitatory post-synaptic potentials (EPSPs) (Fig. 9). Exposures of the periphery to N_2 gas evoked, after a latency of approximately 30-40 sec, a slow depolarization of about 8 mV. Re-exposure of the periphery to flowing air gave a restoration of the normal membrane potential (Fig. 9).

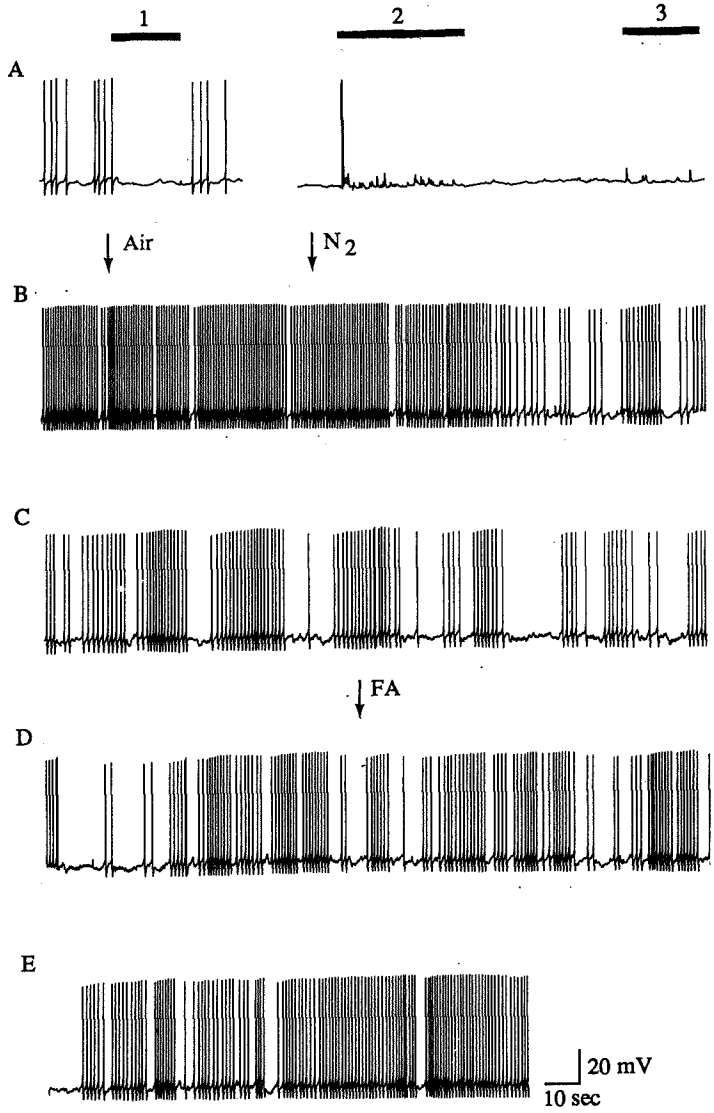


Fig. 10. Recording of RPeD1 during stimulations of the mantle edge (1); pneumostome area (2), and wall of the lung cavity (3). B-E. Continuous recording of RPeD1 during exposures to air (Air), N_2 gas (N_2) and flowing air (FA). A and B-E are from different preparations.

(c) RPeD1

RPeD1 is a giant neurone (diameter: ± 160 µm) in the right pedal ganglion near the statocyst. As noted by Benjamin and Winlow (1981) in an isolated CNS, the cell had a rather regular firing pattern. Mechanical stimulation of the mantle edge produced inhibitory post-synaptic potential (IPSPs), stimulation of the pneumostome area and the wall of the lung cavity produced EPSPs which occasionally gave rise to an action

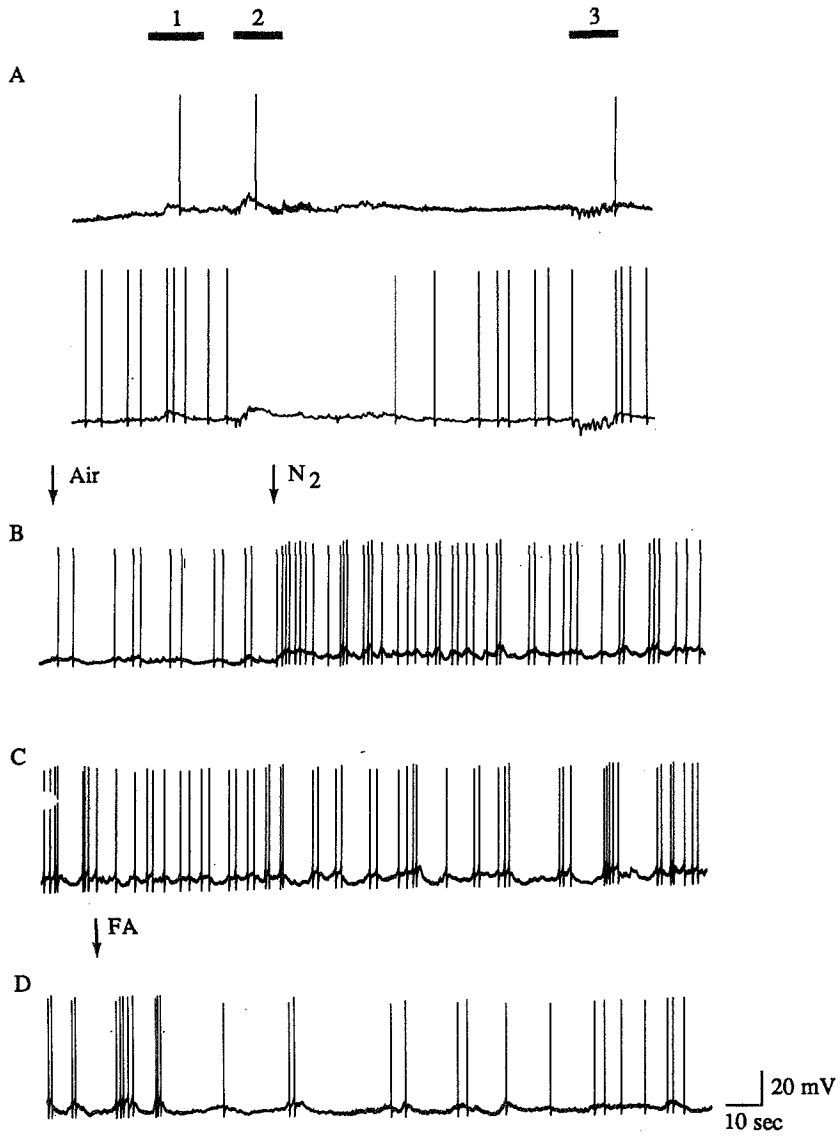


Fig. 11. A. Simultaneous recordings of a B cell (upper) and F cell (lower) during mechanical stimulation of the mantle edge (1); pneumostome area (2), and wall of the lung cavity (3), B, C and D. Recordings of a B cell during exposure of the mantle area to air (Air), N_2 gas (N_2) and flowing air (FA). B, C and D are continuous recordings.

potential (Fig. 10A). Exposure of the mantle to N_2 gas produced a slight hyperpolarization and a decrease in the firing frequency of the neurone. The latency of the response was about 50 sec. Upon exposure to flowing air, the membrane potential and firing frequency increased again (Fig. 10 B - E).

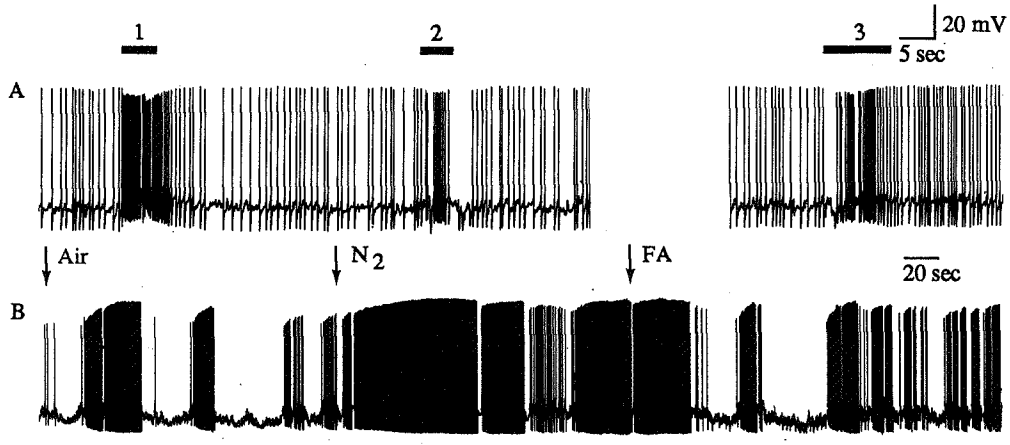


Fig. 12. Recordings of a G cell. A. During mechanical stimulation of the mantle edge (1); pneumostome rea (2), and wall of the lung cavity (3). B. During exposures of the mantle area to air (air), N_2 gas (N_2) and flowing air (FA). A and B are from different preparations.

Responses of neurones from identified groups

(a) Neurones of the B and F group

The B group cells are found in the left anterior part of the right parietal ganglion, the F group cells lie in a cluster in the left caudal part of the visceral ganglion (Benjamin and Winlow, 1981). Cells of both groups fired actions potentials at a low frequency (0.1-2 Hz). This agrees with the findings of Benjamin and Winlow (1981). In some of our preparations these cells were also found occasionally to be silent. The B and F cells have a common inhibitory synaptic input (Benjamin and Winlow, 1981), which was used as a means for identification. Our experiments showed that B- and F-group cells had similar responses to mechanical stimulations as well as to changes in the P_{O_2}. Mechanical stimulations of the wall in the lung cavity produced IPSPs, whereas stimulation of the pneumostome area and the mantle edge produced EPSPs (Fig. 11A).

About half of the B and F cells (a total of about 30 penetrations were made) did not respond to P_{O_2} changes over the mantle area. Responsive as well as non-responsive cells could be found in the same preparations among the B- and F clusters. Probably these clusters are functionally heterogeneous groups of cells. The responsive cells showed a depolarization of approximately 4 mV and an increase in their firing frequency upon exposure of the mantle area to N_2 gas. The latency of the response was about 15 sec. Exposure to flowing air resulted in a restoration of the normal activity of these cells (Fig. 11B-D).

Table 1. Responses of identified cells or groups of cells which did not respond to external P_{O_2} changes to mechanical stimulation of the mantle edge and the pneumostome area.

Cell type	Response upon stimulation of Mantle edge	Pneumostome area	Lung wall
YC	H	H	H
LYC	H	H	H
RPD_1	E	E	E

YC: yellow cells; LYC, light yellow cells; RPD1: right parietal dorsal 1. H: hyperpolarization; E: excitation consisting of depolarization with action potentials.

(b) Neurones of the G group

The cells of the G cluster are located in the dorsal part of the visceral ganglion, between the F cluster and the Yellow Cells (Fig. 5). These cells are easily recognizable because they are small and less whitish than the surrounding cells. In isolated brain preparations these cells are silent (Benjamin and Winlow, 1981); in our semi-intact preparations, however, they fired irregularly (Fig. 12). In the present study it appeared that mechanical stimulation of the mantle edge and the area around the pneumostome evoked a depolarization and an increased frequency of action potentials, this was (especially in the pneumostome area) followed by a period of inhibition lasting several seconds. Stimulation of the wall of the lung cavity produced a slight inhibition, followed by a burst of action potentials (Fig. 12A, B).

Exposures of the mantle are to N_2 gas produced (after a latency of 20-30 sec) a slight depolarization and a considerable increase of the firing frequency. Exposure to flowing air restored the normal firing rate again (Fig. 12B).

(c) Neurones which did not respond to P_{O_2} changes over the mantle area

Many neurones did not respond to P_{O_2} changes over the mantle area. Among these were identified as well as previously not identified neurones. Many of these neurones received synaptic input upon mechanical stimulation of the mantle. Table 1 shows a summary of the previously identified neurones which did not respond to changes in the external P_{O_2} over the mantle area, but which did respond to mechanical stimulation of the same area. These observations indicate that there are mechano-receptive elements in the mantle area

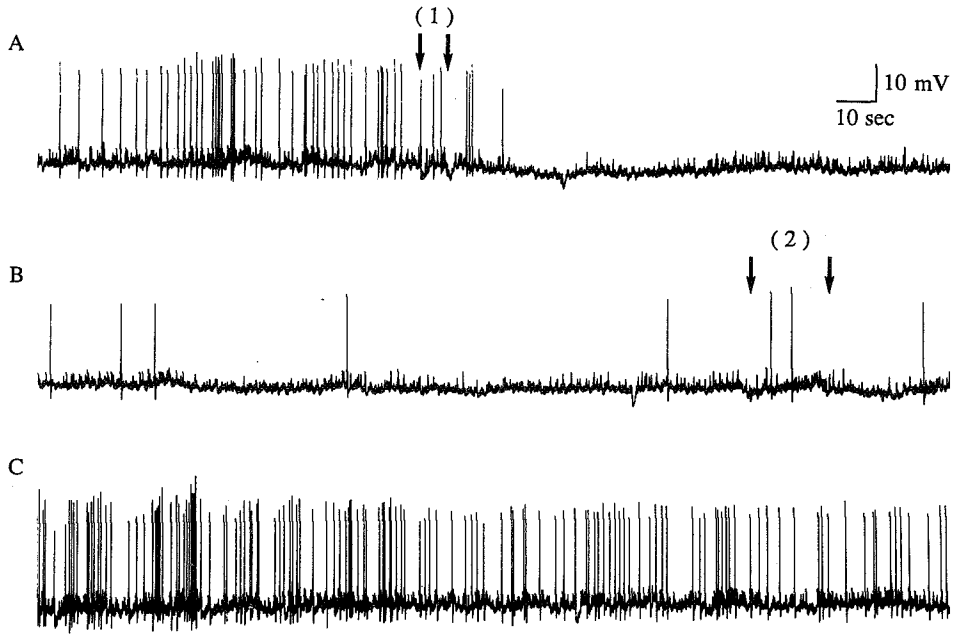


Fig. 13. Responses of a statocyst sensory cell to replacement of Ringer solution with high PO_2 with Ringer with low PO_2 (arrows 1) and again with Ringer with high PO_2 (arrows 2). A - C, continuous recording.

that are not activated by changes in the P_{O_2}. Moreover, the fact that the Light Yellow cells (LYCs) and Yellow Cells (YCs) did not respond to P_{O_2} changes over the mantle area, although they have axons in the mantle nerves (Swindale and Benjamin, 1976; van Swigchem *et al.*, 1981a), indicates that the changes in the P_{O_2} used in the present study did not affect axons of central neurones directly. Therefore, the input on central neurones observed during P_{O_2} changes over the mantle area is most likely to originate from specific afferents synapsing on these neurones.

Responses of statocyst sensory cells to changes in PO_2 in the lung - mantle area

Previously, Janse (1981) has suggested that efferent innervation might play a role in the control of sensitivity of statocyst sensory cells during geotaxis. Therefore, in preparations consisting of the CNS connected to the mantle with the mantle nerves, the responses of the statocyst sensory cells were studied upon exposures of the mantle area to Ringer solution with high and low oxygen content and to air or N_2-gas. Cells were penetrated near the equator of the statocyst, but in different areas.

In general the statocyst sensory cells were extremely sensitive to small fluid movements over the body wall. Some, but not all of the statocyst cells responded to exposure of the mantle area to Ringer solution with low PO_2 with a hyperpolarization.

During the hyperpolarization the firing frequency of the cells was considerably decreased. It was conspicuous that the membrane fluctuations stayed present (Fig. 13). Recovery of the membrane potential and firing frequency were obtained with Ringer solution with high PO_2. Similar results were obtained upon exposure of the mantle area to N_2-gas or to air, respectively. Responses upon PO_2 changes in Ringer or air were obtained in 6 preparations.

DISCUSSION

The nature of the responses of central neurones to changes of the external P_{O_2}

The present study shows that in the central nervous system of *Lymnaea* neurones exist that are sensitive to changes in the P_{O_2} over the mantle and opened lung cavity. Excitatory, as well as inhibitory responses were found upon lowering the external P_{O_2}. In this respect the responses of the present study resemble the responses of neurones upon direct exposure to changes in the P_{O_2}, that is responses to changes in the saline bathing the neurones (Kerkut and York, 1969; Steffin, 1975; Coyer, 1983; Coyer *et al.*, 1983). In other aspects there were, however, differences. All neurones of the present study responded in a reversible way and with a relatively short latency. In the case of direct exposure to low PO_2 long lasting exposures (1-2 hr) were used; moreover, not all of the responses described by the previous authors were reversible.

Several neurones of *Lymnaea* which were shown in the present study to be sensitive to the external P_{O_2} have axons in several mantle nerves. This has been demonstrated for VD_1 and RPD_2 (Boer *et al.*, 1979; Kerkhoven *et al.*, 1991), for $RPeD_1$ (Haydon and Winlow, 1981) and for the B cells (unpublished results). Yet there are several arguments in favour of the view that the effects of the external P_{O_2} are mediated via specific receptors. (1) Recordings from mantle nerves showed that only a limited number of neural elements responded with increased activity upon exposure of the mantle area to N_2 gas. This indicates that changes in the P_{O_2} over the lung - and mantle area act selectively on neuronal activity. (2) Several neurones with axons in mantle nerves did not respond to P_{O_2} changes over the mantle area, indicating that the exposures to air or N_2 gas did not affect these neurones directly. (3) It is not likely that the responses are mediated by mechanoreceptors that are activated by changes in PO_2: Many neurones which receive input upon mechanical stimulation of the mantle did not respond to exposures of the mantle area to different P_{O_2}. (4) the present study showed that responses of central neurones upon changes in the P_{O_2} over the mantle area usually differ from responses upon mechanical stimulation of the skin in the mantle and the internal lining of the lung cavity. In a number of cases inhibitory responses were obtained upon exposure to N_2 gas but excitatory responses upon mechanical stimulation of the mantle (e.g. see the

responses of VD_1). This indicates that mechano-receptive elements in the mantle area of *Lymnaea* exist that are not responsive to N_2 gas exposures.

The conclusion that *Lymnaea* has receptors sensitive to P_{O_2} changes is in agreement with results of previous studies in which it has been suggested that *Lymnaea* has O_2-sensitive receptors in the lung and in the external skin (Janse, 1981). The occurrence of receptors which sense the external P_{O_2} has so far only been reported in arthropods (Page, 1973; Crabtree and Page, 1974; Thompson and Page, 1975) and fish (Bamford, 1974; Daxboeck and Holeton, 1978). This type of response of the O_2-sensitive elements in the periphery of *Lymnaea*, which consists of an increase in the neural activity upon lowering the P_{O_2}, resembles that of a particular class of receptors (Class II) in *Limulus* (Crabtree and Page, 1974), and that of the carotid body of higher vertebrates (Biscoe, 1971). The latency of the response of central neurones to P_{O_2} changes in the skin of *Lymnaea* was 15-50 sec. In the pallial nerves the latency of the response to P_{O_2} changes was of the same order of magnitude. In *Limulus*, similar latencies were found in the peripheral O_2-sensitive elements (Thompson and Page, 1975).

From the central neurons that were studied, the function is known of only the statocyst cells: they are used for orientation in the gravitational field (Janse, 1982). The response of some of the statocyst cells to a change in PO_2 in the mantle area suggests that indeed, these cells receive efferent input originating from O_2 chemosensitive structures in this area. This may at least partly account for the effects of O_2 availability on orientation of *Lymnaea*. In O_2 rich water, immediately after breathing, *Lymnaea* tends to move to the bottom of the water. In contrast, in O_2 poor water as well as after prolonged submergence in O_2 rich water, *Lymnaea* tends to move to the water surface (see Chapters 2 and 3). Janse (1982) has shown that *Lymnaea* uses mainly its statocysts for both types of orientation. Moreover, it has been suggested that different types of statocyst cells might be involved in the two types of gravity orientation (Geuze, 1968; Janse, 1981, 1982). The cells which were found in the present study to be inhibited by exposure of the mantle area to low PO_2 may be involved in positive geotaxis. In view of the foregoing one would also expect to find cells with excitatory responses upon exposures of the mantle area to low PO_2. We did not observe such cells. This may have been due to the fact that only cells in a limited part (the dorsal half) of the statocyst could be penetrated.

Indications of efferent innervation of statocysts have also been obtained in other gastropods (*Hermissenda* , Alkon *et al.*, 1978; *Aplysia*, Salánki and Jahan Parwar, 1985; *Arion*, *Limax* and *Helix* , Wolff , 1970), in cephalopods (Budelman and Bonn, 1982; Williamson, 1986) and in vertebrates (Goldberg and Fernandez, 1980). The function of efferent innervation is in general related to an alteration of afferent responses (Williamson, 1986). In *Lymnaea* efferent control of the statocysts might play a role in modulation of gravity orientation by O_2 chemosensitivity.

It is, as yet, not possible to tell which types of behaviour or physiological processes are regulated by the other neurones that appear to be involved in the processing of O_2 chemosensory information in the brain of *Lymnaea*. In the foregoing chapters, we demonstrated that pneumostome movements at the water surface are influenced by changes in ambient PO_2. These movements are, therefore, among the potential candidates that are controled by the neurones which activity was demonstrated to be changed by changes in the PO_2 over the lung- and mantle area. As may be expected, there are, however, other types of activity that are known to be affected by O_2 availability as well (*e. g.* egg-laying behaviour (Timmermans, 1959; ter Maat *et al.*, 1983), ionic regulation (de With, 1980), metabolic activity (Geraerts, 1975; de Zwaan *et al.*, 1976). A major further step would be to identify which processes and behaviours are controled by the neurones that in the present study were demonstrated to respond in a specific way to changes in external PO_2. The purpose of the next chapter is to examine whether these neurones play a role in control of ventilatory movements of the pneumostome.

REFERENCES

Bamford O.S. (1974) Oxygen reception in the rainbow trout (*Salmo gairdnerii*). Comp. Biochem. Physiol. **48A**: 69-76.

Benjamin P.R. and Winlow W. (1981) The distribution of three wide-acting synaptic inputs to identified neurons in the isolated rain of *Lymnaea stagnalis* (L.). Comp. Biochem. Physiol. **70A**: 293-307.

Biscoe T.J. (1971) Carotid body: structure and function. Physiol. Rev. **51**: 437-495.

Boer H.H., Schot L.P.C., Roubos E.W., ter Maat A., Lodder J.C., Reichelt D. and Swaab D.F. (1979) ACTH-like immunoreactivity in two electronically coupled giant neurones in the pond snail *Lymnaea stagnalis*. Cell Tiss. Res. **202**: 231-240.

Bogerd J. Generation of neuropeptide diversity by alternative splicing in two giant neurons, VD1 and RPD2, of the mollusc *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.

Coyer P.E. (1983) Responses of regularly-firing *Aplysia* R3-R13 neurones to normoxIa and hypoxia. J.Exp. Biol. **107**: 1-8.

Coyer P.E., Halsey Jr. J.H. and Strong E.R. (1983) Reversible changes in the intracellular potassium ion activities and membrane potentials of *Aplysia* L2-L6 neurones in response to normoxia and hypoxia. J. Exp. Biol. **102**: 79-92.

Crabtree R.L. and Page C.H. (1974) Oxygen-sensitive elements in the book gills of *Limulus polyphemus*. J. Exp. Biol. **60**: 631-639.

Daxboeck C. and Holeton G.F. (1978) Oxygen receptors in the rainbow trout, *Salmo gairdnerii*. Can. J. Zool. **56**: 1254-1259.

Geraerts W.P.M. (1975) Studies on the endocrine control of growth and reproduction in the hermaphrodite pulmonate snail *Lymnaea stagnalis*. Thesis Vrije Universiteit, Amsterdam.

Geuze J. J. (1968) Observations on the function and the structure of the statocysts of *Lymnaea stagnalis*. Neth. J. Zool. **18**: 155-204.

Haydon P.G. and Winlow W. (1981) Morphology of the giant dopamine-containing neurone $RPeD_1$, in *Lymnaea stagnalis* revealed by Lucifer Yellow CH. J. exp. Biol. **94**: 149-157.

Janse C. (1974) A neurophysiological study of the peripheral tactile system of the pond snail *Lymnaea stagnalis* (L.). Neth. J. Zool. **24**: 93-161.

Janse C. (1981) The effect of oxygen on gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **142**: 51-59.

Janse C. (1982) Sensory systems involved in gravity orientation in the pulmonate snail *Lymnaea stagnalis*. J. Comp. Physiol. **145**: 311-319.

Janse C. (1983) The function of the statocyst sensory cells in *Aplysia limacina*. J. Comp. Physiol. **150 A**: 359-370.

Kerkhoven R. M., Croll R. P., van Minnen J., Bogerd J., Ramkema M. D., Lodder H and Boer H. H. (1991). Axonal mapping of the giant peptidergic neurons VD1 and RPD2 located in the central nervous system of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain research **556**: 8 - 16.

Kerkut G.A. and York B. (1969) The oxygen sensitivity of the electrogenic sodium pump in snail neurones. Comp. Biochem. Physiol. **28**: 1125-1134.

McKee A. E. and Wiederhold M. L. (1974) *Aplysia* statocyst receptor cells: fine structure. Br. Res. **81**: 310-313.

ter Maat A., Lodder J.C. and Wilbrink M. (1983) Induction of egg-laying in the pond snail *Lymnaea stagnalis* by environmental stimulation of the release of ovulation hormone from the caudo-dorsal cells. Int. J. Repr. **6**: 239-247.

Page C.H. (1973) Localization of *Limulus polyphemus* oxygen sensitivity. Biol. Bull. **144**: 383-390.

Steffin M. (1975) An oxygen dependent current in *Aplysia* neurons: an approach to the question of cellular hypoxic adaptation. Comp. Biochem. Physiol. **52A**: 691-699.

van Swigchem H., Lodder J.C. and de Boer T.S.T. (1981a) Location and properties of the axons of neurosecretory bursting pacemaker cells in *Lymnaea stagnalis* (L.). Proc. Kon. Ned. Akad. Wet. **C84**: 49-62.

Swindale N.V. and Benjamin P.R. (1976) The anatomy of neurosecretory neurones in the pond snail *Lymnaea stagnalis* (L.). Phil. Trans. R. Soc. Lond. **274**: 169-202.

Thompson C. and Page C.H. (1975) Nervous control of respiration: oxygen-sensitive elements in the prosoma of *Limulus polyphemus*. J. Exp. Biol. **62**: 545-554.

Timmermans L.P.M. (1959) Stimulation of oviposition in some land- and freshwater snails. Proc. Kon. Ned. Akad. Wet. **C63**: 363-372.

Wendelaar Bonga S.E. (1970) Ultrastructure and histochemistry of neurosecretory cells and neurohaemal areas in the pond snail *Lymnaea stagnalis* (L.). Z. Zellforschung **108**: 190-224.

de With N.D., Witteveen J. and van der Woude H.A. (1980) Integumental Na^+/H^+ and Cl^-/HCO_3^- exchanges n the freshwater snail *Lymnaea stagnalis*. Proc. Kon. Ned. Akad. Wet. **C83**: 209-215.

Wolff H. G. (1970) Efferente Aktivität in den Statonerven einiger Landpulmonaten (Gastropoda). Z. vergl. Physiol. **70**: 401-409.

Wolff H. G. (1975) Statocysts and geotactic behaviour in gastropod molluscs. Fortschr. Zool. **23**: 63-84.

de Zwaan A., Mohamed A.M. and Geraerts W.P.M. (1976) Anaerobiosis in *Lymnaea stagnalis*. Neth. J. Zool. **26**: 549-557.

CHAPTER 7

NEURAL CONTROL OF PNEUMOSTOME MOVEMENTS IN THE FRESHWATER PULMONATE SNAIL, *LYMNAEA STAGNALIS*

SUMMARY

Neurons in the CNS of *Lymnaea stagnalis* are described that are involved in control of pneumostome movements. These neurons differ from each other in the following aspects: 1) the type of pneumostome movement induced and 2) the sensitivity to sensory stimulation. Based on these differences, the neurons can be considered to be involved in control of pneumostome movements that are part of one of the following behaviours: respiration, escape and withdrawal. The results indicate that neurons that are involved in control of functionally different types of pneumostome movements act largely independent from each other.

Spike activity of putative (pre)motor - neurons that are involved in control of respiration is mainly determined by two wide acting synaptic inputs. The activity of these two inputs is in turn affected by spike activity in two identified peptidergic giant neurons, VD1 and RPD2. Effects of O_2 chemosensory input, addressed to VD1 and RPD2 can explain the effects of changes in PO_2 on respiration.

In addition to central mechanisms involved in control of pneumostome movements, indications have been obtained for the involvement of a peripheral neural network: if the connections with the central nervous system have been cut, the pneumostome is still capable of coordinated opening- and closure movements. The effects of PO_2 changes in the lung- and mantle area on pneumnostome movements are, however, mediated by the central nervous system.

INTRODUCTION

Behavioural studies have shown that in *Lymnaea*, pneumostome movements are not only constitutive of respiratory behaviour, but occur also as part of escape- and withdrawal behaviour (chapter 2). Respiratory pneumostome movements consist of opening- and closure movements, carried out at the surface of the water. They permit gas exchange between the lung cavity and the atmosphere. Pneumostome opening movements can be initiated by surfacing of the animal, and are terminated again by subsequent submergence. The number and duration of pneumostome opening movements during a surfacing period

were shown to depend on the environmental partial pressures of O_2 and CO_2 (PO_2 and PCO_2, respectively). From the results presented in Chapter 2, we concluded that in *Lymnaea* migrations between the bottom and the surface of the water are primarily dependent on the PO_2 of the ambient water; frequency and duration of respiratory pneumostome movements at the surface mainly depend on PO_2 of the inspired air. In this model, restoration of the PO_2 (in lung cavity or adjacent circulatory system) determines to a large extent termination of a respiratory bout and return to the lower depths of the water.

Pneumostome movements during an escape reaction consist of rapid opening, immediately followed by closure. Opening of the pneumostome serves to expel air from the lung cavity during retraction of the animal into the shell, and results in decreased buoyancy of the animal. As a result, the animal sinks to the bottom of the water. It can be induced by strong mechanical stimuli of any part of the skin and by shadow stimuli. Pneumostome movement during withdrawal consists of closure, and can be elicited by mild mechanical stimulation of the pneumostome area and adjacent mantle area (chapter 2).

These behavioural observations suggest that pneumostome movements are under multi-modal sensory control, and that different types of sensory stimulation elicit functionally different types of responses of the pneumostome. With respect to neural control mechanisms, this implies that either a single network is capable of generating these functionally different types of pneumostome movements, or multiple networks exist, that act in parallel (for reviews see Croll and Davis, 1987; Davis, 1985; Getting and Dekin, 1985). In order to distinguish between these two possibilities and to gain insight in neural control mechanisms that are involved in mediating these responses, we studied pneumostome movements and their associated neural control in a semi-intact preparation. We have identified central neurons that have properties which are appropriate for a regulatory role in each of the functionally different types of pneumostome movements. Our data suggest that neurons that are involved in control of functionally different types of pneumostome movements act largely independent from each other, instead of being part of a single network, capable of producing different outputs.

MATERIALS AND METHODS

Animals

Adult specimens of *Lymnaea stagnalis* were used, shell length *ca.* 30 mm. They were bred under standard laboratory conditions at a 12 h - 12 h dark - light regime, and fed lettuce every other day.

Preparations

Prior to dissection, animals were anaesthetized with an injection of *ca.* 1 ml of 30 mM $MgCl_2$ into the foot. The shell was removed and the animal was pinned down in a petri dish, lined with Xantopren (Bayer) and filled with saline. A medial incision was made in the dorsal skin, running from the level between the two tentacles up to just anteriorly to the mantle. There, at right angles, the skin was cut at both sides down to the foot. The two skin halves were folded apart and pinned down. Next, the buccal mass was pulled anteriorly and pinned down. Then, the penis, vas deferens and prostrate gland were removed. The oesophagus was cut anteriorly, leaving a short end attached to the buccal mass, and posteriorly, where it enters the diaphragm. Before the oesopagus could be removed, nerve and connective tissue, running to the salivary glands were cut. The nerves, originating in the visceral and parietal ganglia were left attached to the lung-mantle area. All other nerves were cut. For preparation of the isolated CNS, the nerves from the visceral ganglion and parietal ganglia were also cut, and the CNS was transferred to a recording chamber, lined with Xantopren and filled with saline.

For the semi-intact preparation, dissection was continued as follows. The lung - mantle area was isolated from the foot. The connective tissue, covering the visceral mass was removed with forceps and the visceral mass, including the ovotestis, was removed from the lung-mantle area. Care was taken not to damage the lining of the lung cavity, which is very thin and delicate in this region. Finally, the lung cavity was cut open dorsally, by a transverse incision. The incision was made just anterior to the kidney, running from the pericard (which was left intact) at the left side, to a spot at a distance of *ca.* 2 mm from the inner folds of the pneumostome at the right side. Thus, what remained after dissection was the mantle edge, pneumostome and lung cavity, containing the pericard and kidney, oothecal gland and bursa copulatrix, connected to the CNS by the visceral nerves. The preparation was transferred and pinned down in a recording chamber, lined with Xantopren and filled with saline. The CNS was separated from the peripheral structures by a partition, with nerves running underneath. The separation was made air-and water tight with grease. The compartment containing the lung - mantle area (peripheral compartment) and the compartment containing the CNS (central compartment) each had a volume of *ca.* 4 ml. Unless mentioned otherwise, the peripheral compartment was filled with saline.

Application of stimuli

The separation of the recording chamber into two compartments allowed removal of the saline and exposure of the lung cavity to air or N_2, without disturbing the CNS.

The lung cavity was exposed to air by removing the saline in the peripheral compartment. Exposure of the nerves to air was prevented by covering them with grease. After removal of the saline, the lung cavity could be exposed to a flow of a water -

saturated mixture of air and nitrogen. The speed of the gas flow was *ca.* 250 ml. min-1, controlled by mass flow controllers (Brooks Instruments). During exposure of the lung cavity to gas, the peripheral compartment was covered with a glass slid or with plastic foil.

Mechanical stimuli of different parts of the peripheral structure were given by hand, using a glass rod or a brush. Light-off stimuli were presented by turning off the microscope lamp.

Determinations of PO_2

The PO_2 was measured with a polarographic oxygen electrode, connected to an amplifier (Instech Laboratories). The signal was fed to a pen recorder (Gould). The electrode was calibrated with saline, equilibrated with pressurized air (150 Torr) or a solution of 0.01 M $Na_2S_2O_4$ (Merck, Darmstadt) (0 Torr).

Recording of pneumostome movements

A simultaneous emittor and sensor of infrared light (Bull) was positioned close to the pneumostome. Pneumostome movements resulted in a change of the amount of reflected light, which was measured with the infrared transducer. The trancducer signal was fed to an amplifier and pen recorder. We preferred this optical recording of pneumostome movements over mechanical recordings, for two reasons: 1) mechanical recordings require attachment of a thread to the pneumostome and may thereby cause artifacts; 2) mechanical recordings appeared to be particularly suited for recording pneumostome contractions; they were not very suitable for recording respiratory movements which consist of opening, followed by closure of the pneumostome. However, optical measurement also appeared to impose certain limitations. Although optical measurement could record contractions - as well as respiratory movements, the two movements could not be recorded simultaneously. Moreover, it appeared that the type of the signal (voltage increase or decrease) depended on the position of the sensor relative to the pneumostome. This means that in different preparations the same signal from the transducer could signify either a contraction of the pneumostome or a respiratory pneumostome movement. Therefore, simultaneous visual observation of the preparation was always required to determine the nature of the measured movement. As a result of this, upward or downward deflections of the signal from the infrared transducer shown in this chapter, can signify either contractions or respiratory movements.

The composition of the saline was as follows: $CaCl_2.2H_2O$, 4 mM; $MgCl_2.6H_2O$, 1.5 mM; NaCl, 30 mM; KCl, 1.7 mM; $NaCH_3SO_4$, 10 mM; $NaHCO_3$, 5 mM; HEPES, 10 mM; adjusted to pH 7.8 with 1 N NaOH. The composition of high Mg, low Ca saline was: $CaCl_2.2H_2O$, 1.5 mM; $MgCl_2.6H_2O$, 17 mM; NaCl, 20 mM; KCl, 1.5 mM; $NaHCO_3$, 5 mM; HEPES, 10 mM; similarly adjusted to pH 7.8 with 1 N NaOH.

Electrophysiological recordings

Intracellular recordings were made with glass micro pipettes with a resistance of 10 - 50 MΩ, filled with 0.5 M KCl. Extracellular recordings were made with glass suction electrodes. Conventional electrophysiological apparatus was used for recording, storing and displaying signals. The recording amplifiers permitted current injection via the recording electrodes.

Staining procedures

Lucifer Yellow (LY) was injected into the neurons by applying square wave, hyperpolarizing currents of 2 nA and 400 ms duration at a frequency of *ca.* 2 Hz during 30 min. Subsequently, the ganglion was transferred to a dish, containing 4 % formaline in a 0.1 M acetate buffering solution, pH 4.0. After 30 min, the preparation was dehydrated in the same buffering solution with increasing concentration of alcohol (70, 80, 90, 96 and 100 %). Then the tissue was cleared in methyl-salicylate. The preparations were viewed under a fluorescent microscope.

Cobalt backfillings of nerves were done by sucking the nerves into glass electrodes, and replacing the saline in the electrode with 1 M $CoCl_2$ in deionized water. Pretreatment of the nerve endings with bidest for *ca* 3 min resluted in less variability in staining of central somata among different preparations. Possibly the osmotic shoc prevented clotting of the nerve endings and thus facilitated retrograde transport of the $CoCl_2$. The preparations were left at 4 ^{o}C for 24 h. Next the preparation was rinsed 3 times with saline, and a solution containing 20 % $(NH4)_2S$ was added, resulting in precipitation of CoS. Fixation, dehydration and clearing were carried out as described above.

Identification of central neurons

For identification of central neurons, located on the dorsal surface of the visceral and right parietal ganglia we used the maps and criteria, of Benjamin and Winlow (1981). We used the same nomenclature as introduced by these authors. Based on their description, we could recognize giant neurons VD1, VD2, VD3, RPD1 and RPD2, and neurons from the A, B, F and G group, as well as the Yellow Cells. We could not, however, distinguish between H, I, J and K cells, as we could not demonstrate synaptic input on these neurons from the giant neuron RPeD1 which is used as a means for identification of these neurons (Benjamin and Winlow, 1981). In addition to these neurons, we will present data on neurons that have not been identified previously. In these cases, identification criteria consisted of causal or correlated relationships between spike activity of the relevant neurons and pneumostome movements.

RESULTS

In the present study, three types of preparations were used: 1) lung- mantle area connected to the CNS, 2) lung- mantle area isolated from the CNS, and 3) the isolated CNS. The first preparation was used to study central control of pneumstome movements and sensory input from this area to the CNS. The second preparation was used to determine peripheral components of pneumostome control. The third preparation was used in addition to the first, to study connections between central neurones, associated with control of pneumostome movements.

Pneumostome movements in semi-intact preparations connected to the CNS

In this section, movements of the pneumostome that were observed in the preparation will be described and compared with those, observed in the intact animal. In the reduced preparation clearly recognizable, complete opening- and closure movements of the pneumostome could be observed. As compared to the intact animal, however, the movements usually were somewhat less vigorous. Three types of pneumostome movements are distinguished:

1) Pneumostome contractions: the pneumostome is closed and contracts at more or less regular intervals, resulting in flattening of the pneumostome against the mantle edge.

2) Respiratory movements of the pneumostome, consisting of complete opening, followed by gradual, usually slow closure

3) Escape pneumostome movements: sudden, complete opening of the pneumostome followed by closure. It occurs as a response to strong mechanical stimulation or shadow stimuli, and differs from respiratory movements as the movement is much faster.

The following account is a description based on observations of more than 50 preparations.

Spontaneous movements

As long as the peripheral compartment was filled with saline, the pneumostome was kept closed. Still, most of the time, it showed rhythmic contractions, which resulted in flattening of the pneumostome against the mantle edge. The interval between two of such contractions varied from 4 to 40 s in different preparations. In the following, these contractions will be referred to as pneumostome contractions.

Pneumostome movements induced by sensory stimulation

Removal of the saline in the peripheral compartment usually induced a respiratory movement: opening of the pneumostome, followed by gradual closure. The pneumostome usually remained open for periods lasting from a few up to 50 s. Several of such respiratory movements could occur in succession. Reduction of the PO_2 in the air

of the peripheral compartment resulted in a substantial increase in the number of respiratory movements. When lowering of the saline level was repeated while the PO_2 of the saline and the gas phase in the peripheral compartment were low, it never failed to induce respiratory movements (as described above, this was not always the case at normoxic conditions). Elevation of the saline level in the peripheral compartment, or gentle mechanical stimulation of the pneumostome or mantle edge while the pneumostome was open, always resulted in termination of the respiratory movement by closure of the pneumostome. When the saline level was elevated while the pneumostome was closed, it induced a slight contraction of the pneumostome.

Gentle mechanical stimulation of the pneumostome or the mantle edge with a glass rod or a small paint brush resulted in a contraction of the pneumostome, consisting of flattening of the outer flap of the pneumostome against the mantle edge. More powerful mechanical stimulation of especially the mantle edge with a stroke stimulus often elicited a rapid, complete opening movement of the pneumostome, immediately followed by closure. This pneumostome movement will be referred to as escape pneumostome movement since it is part of an escape reaction of the animal on threatening stimuli. Within a period of 3 - 4 min after one such response had been elicited, application of a second stimulus of equal or higher intensity failed to evoke the response. An escape pneumostome movement could also be elicited by a shadow stimulus to the preparation. These pneumostome movements that are observed in the semi-intact preparation are quite comparable to those, observed in the intact animal.

With respect to respiratory movements, it was observed that in the intact animal, exposure of the pneumostome to the atmosphere can induce respiratory pneumostome movements (see chapter 2). In the case of the semi-intact preparation, lowering of the saline level in the peripheral compartment probably mimicks this sensory stimulus. As in the intact animal, the stimulus appeared to be a prerequisite for respiratory movements to occur, but did not always induce it. In contrast, respiratory movements always occurred when the pneumostome was exposed to the atmosphere during low ambient PO_2 indicating that interactions probably occur between mechanosensory and chemosensory stimuli. Also the pneumostome contractions were observed in the intact animal, viz. during withdrawal after mechanical stimulation of different parts of the skin. Rapid opening of the pneumostome followed by closure was observed in the intact animal during an escape reaction, involving retraction of the animal into its shell; the pneumostome movement serves to allow expulsion of air from the lung cavity (chapter 2).

Pneumostome movements in preparations isolated from the CNS

In order to study the contribution of the peripheral nervous system (PNS) to the regulation of pneumostome movements, we determined whether spontaneous or induced

movements still occurred, after the connections between the mantle area and the central nervous system were cut. In such preparations, the regularly occurring pneumostome contractions, normally observed as long as the mantle area was kept in saline, were abolished. The outer fold of the pneumostome was no longer kept closely apposed to the mantle. Lowering of the saline level, however, could still induce respiratory movements of the pneumostome. In contrast, decrease of the peripheral PO_2 did not cause the increase of respiratory movements of the pneumostome, as was observed when the connections to the CNS had been left intact. Gentle mechanical stimulation of the pneumostome area still elicited contraction of the pneumostome.

Differences in response upon mechanical stimulation were observed between preparations with the nerves cut close to the CNS or close to the mantle area. When the nerves were cut close to the CNS, gentle mechanical stimulation of the mantle edge resulted in contraction of the pneumostome. Stronger mechanical stimuli induced rapid opening and closure of the pneumostome (escape movement). When the nerves were cut close to the entrance to the mantle area, mechanical stimulation of different intensities of the mantle edge did neither result in contractions nor in escape pneumostome movements. Mechanical stimulation of the pneumostome itself only caused contraction of the pneumostome. Differences in response of the pneumostome to mechanical stimulation, related to the length of the visceral nerves that have been left intact may be due to synaptic connections within the nerves, as demonstrated by Lever *et al.* (1977).

Blocking of central synaptic transmission by bathing the CNS in saline with high Mg^{2+} and low Ca^{2+} had the same effects as cutting the nerves close to the CNS. In these instances, complete recovery of all the responses occurred after wash with normal saline.

These results show that in the absence of the CNS, respiratory movements and contractions of the pneumostome can still occur, as well as escape movements provided that a sufficiently long part of visceral nerves was left attached to the periphery. By contrast, the effects of a peripheral decrease of the PO_2 on respiratory movements of the pneumostome appeared to require the CNS. This also holds for the regularly occurring contractions of the pneumostome.

Stimulation, recording and backfilling of visceral nerves

In order to determine which of the visceral nerves contain axonal projections of central somata, involved in control of pneumostome movements, we electrically stimulated each of the nerves after they had been cut close to the CNS and determined the effect on pneumostome movements. Electrical stimulation of the external right parietal nerve (NPDE) with a single square pulse (0.4 nA, 0.5 s) resulted in contraction of the pneumostome (Fig. 1 A, upper traces). Increase of the stimulus intensity resulted in stronger contraction, which was otherwise identical to the response at lower intensity. A single square pulse applied to the anal nerve (0.4 nA, 0.5 s) also resulted in contraction of

the pneumostome. By contrast, higher stimulus intensity (1.2 nA, 0.5 s) caused rapid opening and closure of the pneumostome (Fig. 1 A, lower trace). The movement resembled pneumostome movement during escape, since opening and closure occurred more rapidly than during respiratory movements. Electrical stimulation of the other nerves was ineffective in eliciting movements of the pneumostome.

Extracellular recordings of the NPDE in preparations where the nerves had been left intact revealed increased activity of a small number (2-4) of elements during contraction of the pneumostome (Fig. 1 B, upper traces). In some preparations we observed increased activity of a few elements of large amplitude in the anal nerve (NA) during respiratory movements of the pneumostome (Fig. 1 B, lower traces). We did not observe activity changes in the NA associated with spontaneously occurring contractions of the pneumostome.

The results of the electrical stimulation of the visceral and parietal nerves show that central neurons involved in control of pneumostome movements, project into the NA and NPDE. The retrograde staining with $CoCl_2$ was used to identify central somata projecting into the two nerves. The numbers of stained neurons, found in the different ganglia after backfilling the NA or the NPDE are presented in Table 1. Most cell bodies stained via the NA were located in the visceral ganglion and the right parietal ganglion. Backfilling of the NPDE primarily stained somata in the right parietal ganglion.

Intracellular recordings

The backfillings revealed that stained cell bodies were primarily found in the VG and RPG. Therefore, intracellular recordings were only made from neurons in these two ganglia in semi intact preparations. In the previous chapter we presented data, indicating the involvement of a number of identified neurons in the processing of O_2 chemosensitive information in the central nervous system in *Lymnaea*. Since respiratory movements of the pneumostome are known to be affected by changes in PO_2, it is conceivable that some of these neurons are involved in control of respiration. In the present study it appeared that from these neurons, the giant neurons VD1 and RPD2 and neurons of the G-group show spike activity which correlates with pneumostome movements. This correlation appeared to be due to synaptic input which VD1 and RPD2 and neurons of the G-group receive in common with still a number of other neurons. Therefore, with respect to neuronal control of respiratory movements of the pneumostome, we focussed our attention in the present study on VD1 and RPD2 and neurons of the G-group, on the possible origin of the synaptic inputs which are responsible for correlation between spike activity and pneumostome movements and on other follower cells of these inputs.

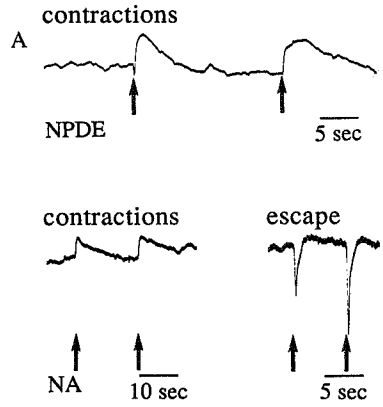


Fig. 1 A. Recordings of pneumostome movements. Electrical stimulation of the NPDE (upper trace) with square wave depolarizing pulses of 0.4 nA resulted in contractions of the pneumostome; between the presentations of the electrical stimuli the pneumostome was in closed position. Stimulation of the nerve resulted in flattening of the pneumostome against the mantle edge. When the stimulus is presented at a time when the pneumostome is open, the pneumostome closes. Similar stimulation of the NA (lower traces) also resulted in contractions of the pneumostome. Stimulation of the NA at higher intensity (1.2 nA) resulted in rapid opening and closure of the pneumostome, resembling pneumostome movements during escape of the animal.

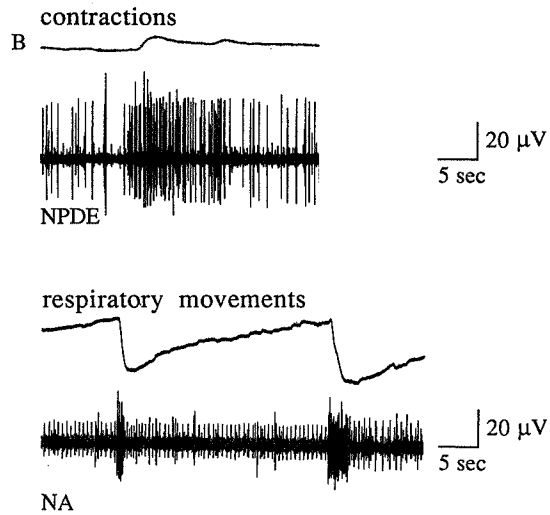


Fig. 1 B. Recordings of pneumostome movements. Upper panel: Recording from the NPDE (lower trace) is associated with increased activity during contractions of the pneumostome (upper trace). Lower panel: Recording from the NA (lower trace) revealed increased activity during the opening movement of the pneumostome (upper trace). These slow opening- an closure movements of the pneumostome resembled respiratory movements of the intact animal.

Inputs C and R

A conspicuous finding was that a relatively large number of neurones, located on the dorsal surface of the visceral ganglion and, to a somewhat lesser extent, the right parietal ganglion, showed correlated activity with either pneumostome contractions, respiratory

Table 1. Distribution and mean number of central somata over the different ganglia stained after back-filling of the anal nerve (NA) and the right parietal external nerve (NPDE) with cobalt. VG: visceral ganglion; RPG: right parietal ganglion; LPG: left parietal ganglion; LPlG: left pleural ganglion; RPlG: right pleural ganglion; LCG: left cerebral ganglion; RCG: right cerebral ganglion; RPeg: right pedal ganglion. RPeD1: giant neuron right pedal dorsal 1.

	NA	NPDE
VG	55	-
RPG	35	15
LPG	6	
LPlG	1-4	
RPlG	1-4	4-5
LCG	2 (anterior lobe)	
RCG		2 (anterior lobe)
RPeG		1 (RPeD1)

pneumostome movements, or both. Thus, pneumostome contractions correlated with excitatory input in VD1 and RPD2, with excitatory input in A - group neurones, with inhibitory synaptic input in a cluster of neurons on the dorsal surface of the visceral ganglion which we have labelled Visceral Dorsal Respiratory (VDR) neurones and excitatory synaptic input in neurons in the H, I, J and K cluster (Figure 2 A - D). The properties of VDR neurons and of VD1 and RPD2 will be described in more detail below; for the moment, I will focus on the characterization of the inputs, associated with contractions and respiratory movements of the pneumostome. We have not identified the neurones from which this input originates with certainty (but see below); in the remainder of this chapter we will refer to this synaptic activity correlating with pneumostome contractions as input C. Once the correlation between the occurrence of this synaptic input and pneumostome contractions had been established and characterized by its effects on follower cells, it could easily be recognized in isolated central nervous systems as well, especially in paired recordings of follower cells. Examples of such recordings are shown in Figure 3. This clearly demonstrates the central origin of this input.

Respiratory movements of the pneumostome correlated with inhibitory input in VD1 and RPD2 (Figure 4 A) and with excitatory synaptic input in VDR neurones (Figure 4 B). This input will be referred to as input R; it could also be observed in the isolated central nervous system, though its occurrence was much rarer than the occurrence of input C. Figure 4 C shows a simultaneous recording of VDR and VD1 in an isolated central nervous system, with input R excitatory in the former and inhibitory in the latter.

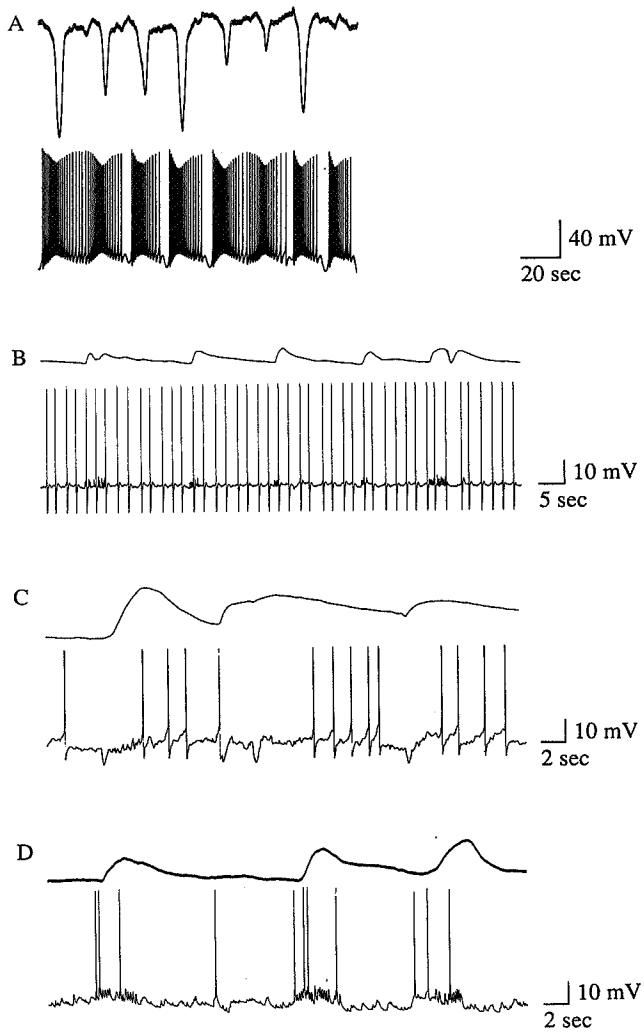


Fig. 2. Simultaneous recordings of electrical activity of central neurons and pneumostome movements. Contractions of the pneumostome correlate with excitation or inhibition of central neurons; such correlations were attributed to the action of a wide-acting synaptic input, called input C. a: VD1, b: A-group neuron, c: VDR neuron, d: neuron in the H, I, J, K cluster.

Thus, to summarize, a relatively large number of neurons in the visceral and right parietal ganglion showed activity which correlated with pneumostome movements. Correlated activity was assigned to the actions of two distinct synaptic inputs. One input was associated with the regularly occurring contractions of the pneumostome (input C). The other input was associated with respiratory movements of the pneumostome (input R). Central neurons that can be used most reliably to monitor the activity of these inputs are (see also Table 2):

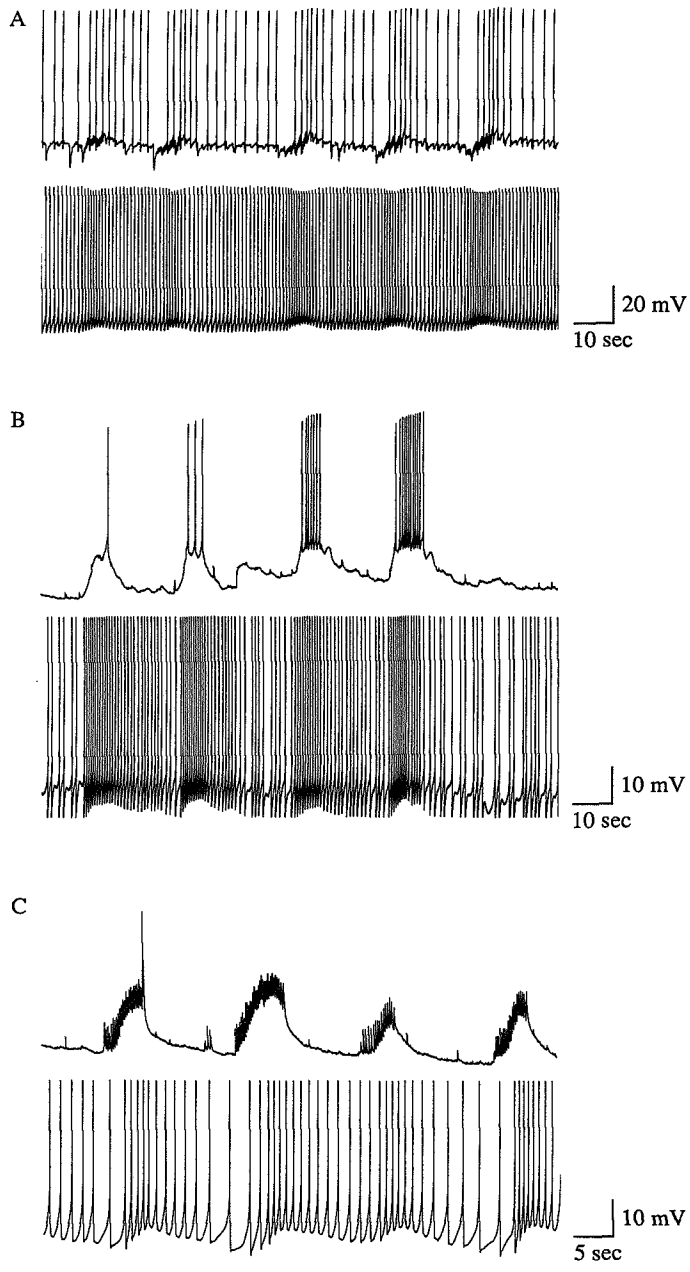


Fig. 3. Paired recordings of central neurons in the isolated central nervous system, showing the simultaneous actions of input C on VD1 and other follower cells. a: excitation of VD1 (lower trace) coincides with inhibition of a VDR neuron (upper trace); b: excitation of VD1 (upper trace) coincides with smooth depolarizations of a cluster I neuron (hyperpolarized); c: excitation of VD1 (lower trace) coincides with excitation of a cluster II neuron, consisting of individual epsp's.

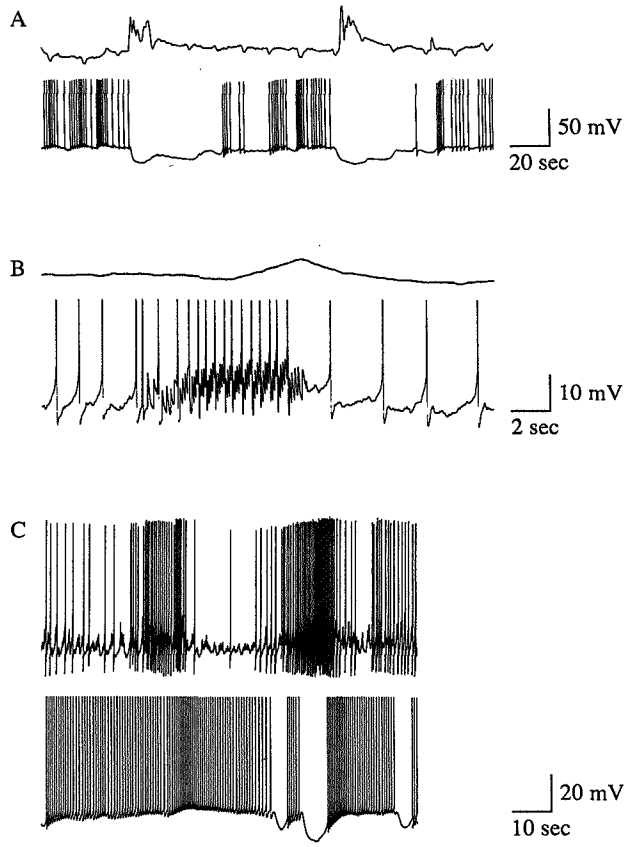


Fig. 4. A and B: Simultaneous recordings of electrical activity of central neurons (lower traces) and pneumostome movements (upper traces). Respiratory movements of the pneumostome correlate with inhibition of VD1 (A) and excitation of a VDR neuron (B). C: paired recording of a VDR neuron (upper trace) and VD1 (lower trace) in the isolated central nervous system, showing the coincidence of excitation of the VDR neuron and inhibition of VD1. Inhibition of VD1 and excitation of VDR neurons, correlating with respiratory movements of the pneumostome were attributed to the action of a synaptic input, called input R.

- VDR neurons - input C is inhibitory, causing hyperpolarization and temporary suppresion of spike activity, input R causes a characteristic burst of EPSP' s with high frequency, resulting in depolarization and increase in spike activity.
- A - group neurons. Particularly when hyperpolarized, input C causes readily recognizable depolarizations of the membrane potential
- VD1 and RPD2 - input C is excitatory, causing depolarizations and concomitant increased firing rate; input R is inhibitory, causing hyperpolarization and suppression of spike activity, sometimes for long periods of time (ranging from 20 seconds up to several minutes).

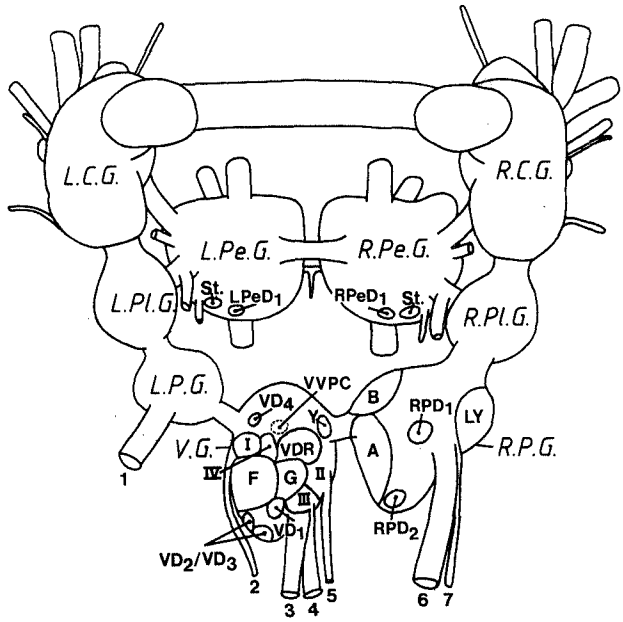


Fig. 5. Schematic representation of the dorsal surface of the CNS of *Lymnaea* (Figure after Slade *et al.*, 1981). The location of previously identified neurons is indicated, using the nomenclature of Benjamin and Winlow (1981), as well as the location of newly described neurons. Abbreviations: LCG and RCG, left- and right cerebral ganglia; LPeG and RPeG, left and right pedal ganglia; LPlG and RPlG, left and right pleural ganglia; LPG and RPG, left and right parietal ganglia; VG, visceral ganglion. St, statocyst. Numbers of nerves refer to the following visceral nerves; 1: left parietal nerve, 2: pallial cutaneous nerve, 3: anal nerve, 4: intestinal nerve, 5: genital nerve, 6: right parietal internal nerve, 7: right parietal external nerve. Groups of neurons - A, B, F, G, Y (yellow cells), LY (light yellow cells), VDR (visceral dorsal respiratory neurons), I, II, III and IV - are indicated, as well as individual neurons: VD (visceral dorsal) 1 - 4; RPD (right parietal dorsal) 1 and 2; LPeD1 and RPeD1 (left- and right pedal 1, respectively), and a neuron located on the ventral surface of the visceral ganglion: VVPC (viscreal ventral pneumostome closer).

In the following we will assess whether neurons receiving input C, input R or both are causally involved in control of pneumostome movements. Our data suggest such a role for VDR neurons and for VD1 and RPD2; the former affect pneumostome movements in a fairly direct way, whereas the latter appear to do so in an indirect way. From a large number of neurones which do receive input C, no causal relationship between spike activity and pneumostome movements could be demonstrated.

VDR neurones: putative motor- or premotor neurones involved in control of respiratory movements of the pneumostome

On the dorsal surface of the visceral ganglion, intermingled between Yellow Cells and cells of the G group (see map, Fig. 5), 3 or 4 neurons are located which, upon suprathreshold electrical stimulation, cause respiratory movements of the pneumostome. Fig. 6 A shows the short latency response of the pneumostome elicited by the stimulation

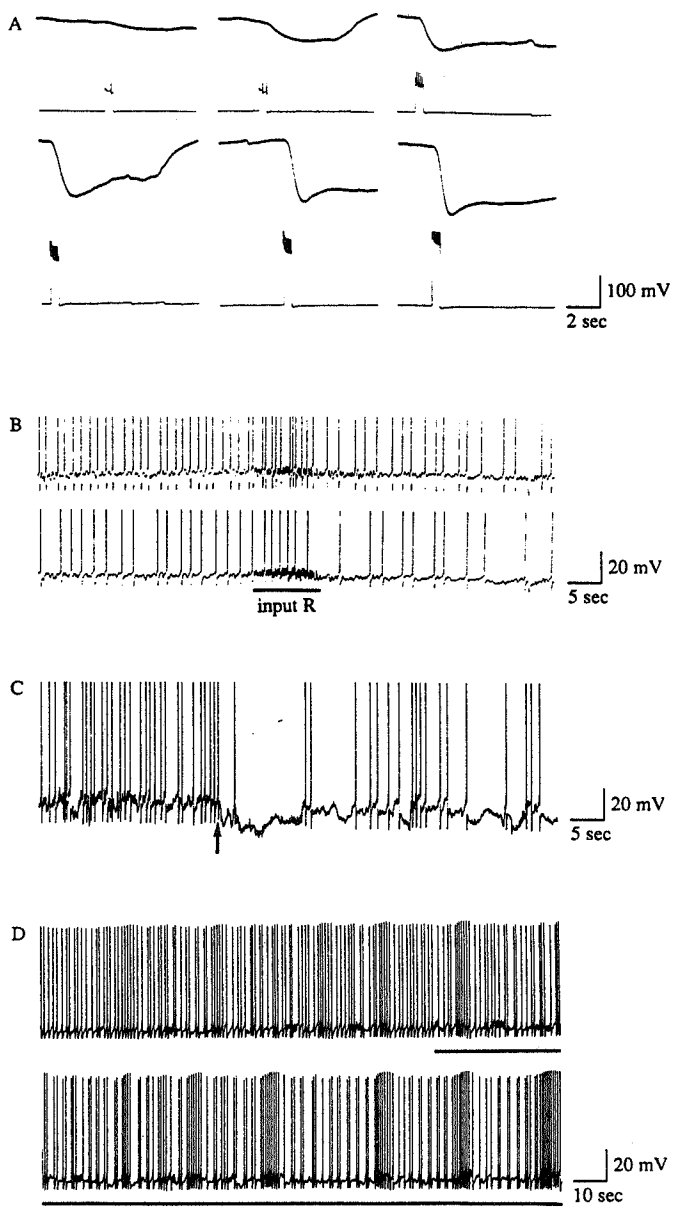


Fig. 6. Recordings of VDR neurons. A. Suprathreshold, squarewave depolarizing pulses of increasing intensity, ranging from 0.5 to 3.0 nA (lower trace) resulted in pneumostome opening movements with increasing amplitude (upper trace). B. Simultaneous recording of two neurons of the VDR cluster revealed common synaptic input, resulting in approximately similar spike activity. C. Elevation of the saline level (arrow) in the compartment containing the lung- and mantle region results in inhibition of a VDR neuron. D. Exposure of the lung - mantle area to air with low PO_2 (bar) resulted in an increase of bursts of high-frequency spike activity spike activity in a VDR neuron. Recordings A - D are from different preparations.

of one of these neurons with increased depolarization of the membrane potential of the neuron. The amplitude, speed and duration of the respiratory movement increase with increasing spike frequency. The neurons receive inhibitory synaptic input during mechanical stimulation of the mantle edge and the pneumostome, resulting in a decrease of firing. Somata of these neurons are pale orange in colour, their diameter varies between 60 - 80 µm. The neurons usually fire irregularly, with a mean frequency of about 0.5 Hz. Application of depolarizing or hyperpolarizing square wave pulses to one neuron did not result in changes of membrane potential of another neuron of the cluster, suggesting that they were not electrotonically coupled. Patterns of spike activity among neurons of this type were similar as a result of common synaptic input (Figure 6 B). As was shown above, the neurons receive inhibitory synaptic input during closure of the pneumostome (input C, see Figure 3) and excitatory input during respiratory movements of the pneumostome (input R; see Figure 4).

If removal of the saline from the peripheral compartment resulted in respiratory movements of the pneumostome, this was accompanied by an increased spike activity in these neurons, due to excitatory synaptic input. Elevation of the saline level resulted in inhibition of VDR neurons (Figure 6 C). Exposure of the lung - mantle area to air with low PO_2 resulted in increased spike activity (Figure 6 D); this was accompanied by increased respiratory movements of the pneumostome. We were not able to demonstrate synaptic input originating from R.Pe.D1 in these neurons. Hence they probably do not correspond to one of the cell types, identified by Benjamin and Winlow (1981) in this part of the CNS. Because of their location and function, we called these neurons Visceral Dorsal Respiratory neurons (VDR).

VDR neurons were recorded from in more than 20 preparations. VDR neurons could be recognized in the isolated CNS, especially in paired recordings with A - group neurons or with VD1 / RPD2 on the basis of the effects of inputs C and R. However, it should be noted that VDR neurons could only be identified with complete certainty in the semi-intact preparation (by their effects on the pneumostome), since in this part of the visceral ganglion many neurons are located which show similar spike activity, but which fail to induce opening of the pneumostome upon depolarization. LY stainings in 11 preparations revealed the presence of 2 or 3 projections in the intestinal nerve (NI), and extensive branching in the region of the visceral ganglion, close to the connective with the right parietal ganglion.

VD1 and RPD2: Putative interneurons involved in control of respiratory pneumostome movements

VD1 and RPD2 are two electrotonically coupled, peptidergic giant neurons, located on the dorsal surface of the visceral- and right parietal ganglion, respectively (Benjamin and Winlow, 1981). Because of their positive immunostaining with antibodies raised against

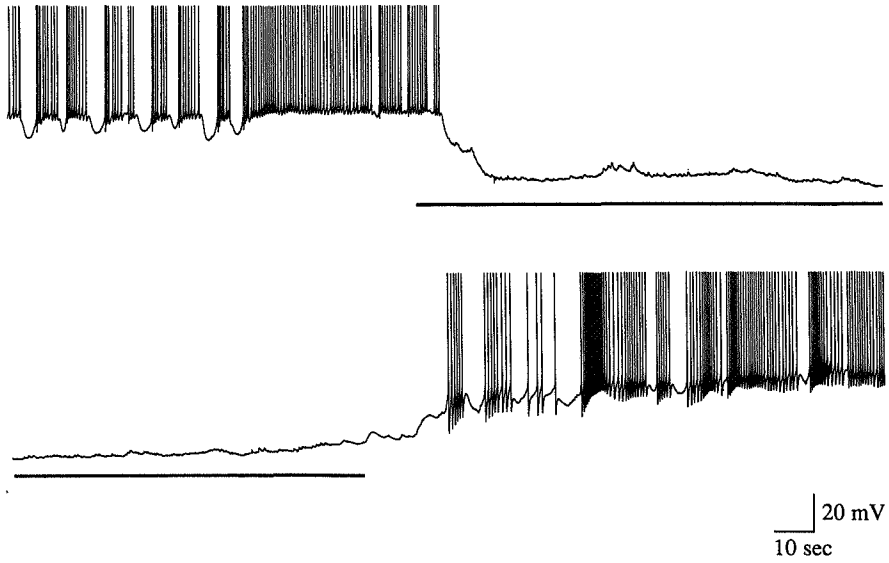


Fig. 7. Exposure of the lung-mantle area to low PO_2, indicated by bar, was accompanied by strong hyperpolarization of VD1 and complete suppression of spike activity. Restoration of the PO_2 in the peripheral compartment was followed by gradual resuming of spike activity in VD1.

vertebrate ACTH, they are also called ACTH cells (Boer *et al.*, 1979). The neurones have axonal projections in the regions of the pneumostome, osphradium and heart (Kerkhoven *et al.*, 1991). They produce cardioactive peptides that show homology with *Aplysia* R15 peptides (Bogerd, 1992). Because of the large soma-diameter and the 1 to 1 spike activity of the two neurons they are easily identifiable; the description, given below is based on recordings of the neurons in more than 60 preparations. As was demonstrated above, spike activity in the two neurons appeared to be closely related to pneumostome movements. VD1 and RPD2 receive excitatory synaptic input during contractions of the pneumostome (input C, see Fig.2) and inhibitory synaptic input during respiratory movements of the pneumostome (input R, see Fig. 4). As was already shown in the previous chapter, exposure of the periphery to low PO_2 causes immediate strong inhibition of the two neurons, resulting in complete suppression of spike activity (Fig. 7). Preliminary results from experiments with selective lesioning of visceral nerves suggest that these effects are mediated by the NA, NPDE, and NPDI: VD1 and RPD2 showed a long lasting hyperpolarization upon lowering of the peripheral PO_2 as long as one of these nerves had been left intact. In preparations where each of these nerves had been cut, and the other nerves were left intact, VD1 and RPD2 did not respond any longer to lowering of the peripheral PO_2. VD1 and RPD2 receive excitatory synaptic input during mechanical stimulation of different parts of the mantle edge and the pneumostome, resulting in depolarization and increased spike frequency (see also chapter 6).

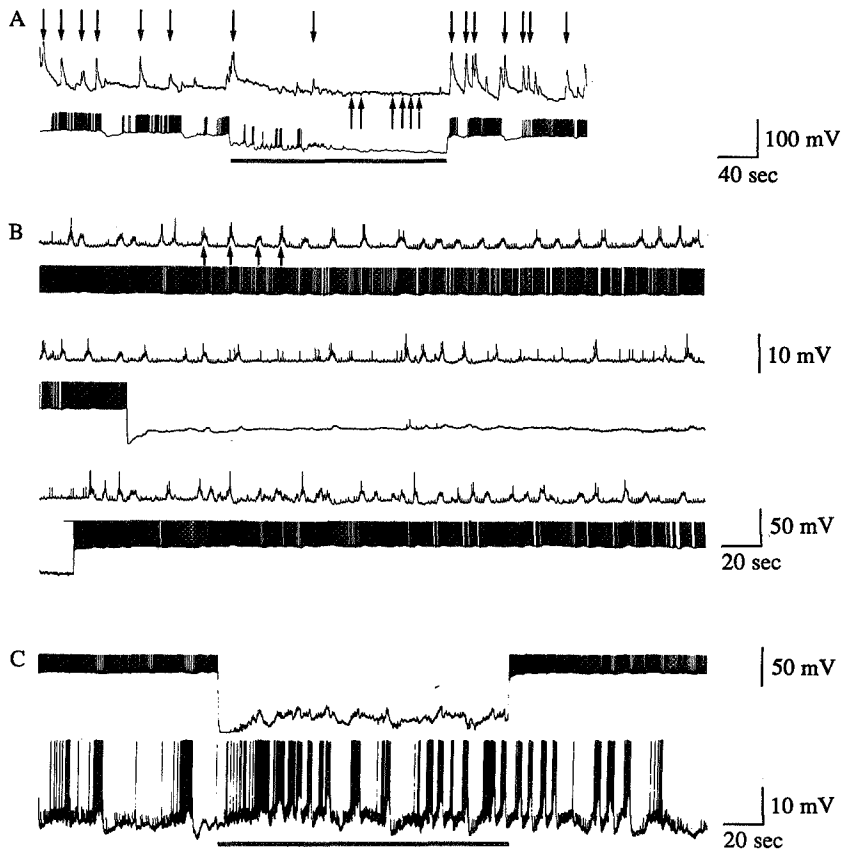


Fig. 8. Effects of prolonged hyperpolarization and suppression of spike activity in VD1 and RPD2. A. Simultaneous recordings of pneumostome movements (upper trace) and VD1 (lower trace). Hyperpolarization indicated by bar. Contractions of the pneumostome (downward arrows) were accompanied by burst activity in VD1. Suppression of spike activity in VD1 resulted in decreased frequency and amplitude of contractions. Initially they still occurred, accompanied by depolarization of VD1, indicative for the occurrence of input C. After prolonged hyperpolarization, contractions of the pneumostome and excitatory synaptic input on VD1 were completely absent. Instead, respiratory movements of the pneumostome occurred (upward arrows) which, in this recording gave rise to only small signals of the sensor. When the hyperpolarizing current was removed, normal spike activity of VD1 and frequency of contractions of the pneumostome were restored again. B. Simultaneous recording of an A-group neuron (hyperpolarized, upper trace) and VD1 (lower trace) in the isolated central nervous system. The three traces are continuous. Input C can be recognized as compound depolarization of the A-group neuron and depolarization and increased spike activity in VD1. Hyperpolarization of VD1 and suppression of spike activity, resulted in a decrease of the frequency of input C, which was restored again when the hyperpolarizing current in VD1 was removed and the neuron was allowed to resume its normal spiking pattern. C. Simultaneous recording of VD1 (upper trace) and a VDR neuron (lower trace) in the isolated central nervous system. Input R can be recognized as burst of activity in the VDR neuron and decreased spike activity in VD1. Hyperpolarization and suppression of spike activity in VD1 (indicated by bar), resulted in increased burst activity in the VDR neuron, indicative of the activity of input R. Removal of hyperpolarizing current in VD1 resulted in its normal spike activity and decrease of burst activity in the VDR neuron to its former level.

So far, our data only suggest a correlation between spike activity in VD1 and RPD2 and pneumostome movements as the result of the actions of inputs C and R. The observations that led us to believe that VD1 and RPD2 are causally involved (albeit indirectly) in control of pneumostome movements, were the effects of prolonged hyperpolarization of VD1 and RPD2, resulting in complete spike suppression during prolonged periods of time, on pneumostome movements:

Application of hyperpolarizing current to the neurons resulted in complete suppression of spike activity after 2 - 3 min. During periods of prolonged hyperpolarization the frequency of regular contractions of the pneumostome was decreased and the frequency of respiratory movements was increased (Fig. 8 A). Similar suppression of spike activity of VD1 and RPD2 also resulted in a decrease of the frequency of input C, as monitored in A - group neurones (Fig. 8 B) and an increase of the frequency of input R, as monitored in VDR neurones (Fig. 8 C). These observations suggest that reciprocal connections exist between VD1 and RPD2 on the one hand, and the neurons, responsible for input C and input R on the other hand.

Since input C and input R appear to play a major role in control of pneumostome movements, we have tried to identify the neurons from which they originate. In spite of numerous efforts, we have not been able to identify them with certainty. Only circumstantial evidence exists that previously unidentified neurons, located in cluster III and cluster IV may constitute the origin of input C and input R, respectively. Although the data are scanty, the importance of inputs C and R in control of pneumostome movements warrants mention of the following results. They should be considered as a possible cue for further identification of the origins of inputs C and R.

Possible origins of inputs C and R

After complete isolation of the visceral ganglion from the rest of the CNS, VD1 and VDR still received inputs C and R. This indicates that inputs C and R both originate in the VG. In keeping with this observation, RPD2 loses inputs C and R after cutting the connection between the VG and the RPG.

In order to identify the neurons that produced inputs C and R, we searched for neurons in the visceral ganglion that were capable of producing excitation or inhibition in VD1 and RPD2, respectively. Two different types of neurons (clusters III and IV, respectively) were found, which affected spike activity in VD1 and RPD2.

Input R: A cluster of neurons, located in the right caudal part of the VG, showed increased spike activity, accompanied by decreased firing rate in VD1 (Fig. 9 A). Depolarization of a neuron of cluster III by current injection, resulting in increased firing rate, was followed by strong inhibition of VD1 (Fig. 9 B). We did not find any other neurons that caused inhibition in VD1 and RPD2; whether these cluster III neurons really constitute the origin of input R remains to be established by assessing their effects on

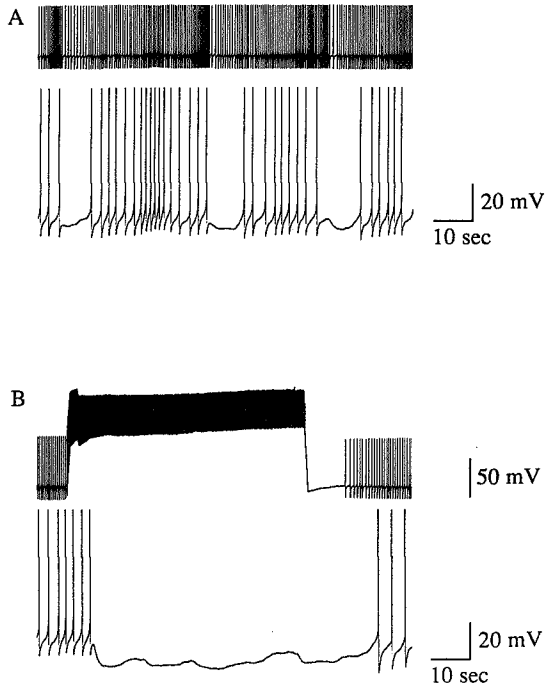


Fig. 9. Paired recording of VD 1 (lower trace) and a cluster III neuron in the isolated central nervous system. A. Inhibition of VD1, which we attributed to the action of input R, was accompanied by increased spike activity of the cluster III neuron. B. Depolarization of a type III neuron (upper trace) was followed by hyperpolarization of VD1 (lower trace).

pneumostome movements and on other follower cells, and the effect of VD1 and RPD2 on the activity of these cells (since we postulated reciprocal connections).

Input C: Cluster IV neurons are located anteriorly to the F-group neurons and have approximately similar soma diameter and colour. In contrast to F neurons, they show bursting activity. These bursts coincide with excitatory actions of input C in VD1. In view of the wide-acting nature of input C, the most likely possibility was that VD1 and cluster IV neurons receive input C in common. Still, in this case there are some indications that cluster IV neurons may themselves be implicated in the generation of input C. Hyperpolarization of VD1 resulted in a gradual decrease of spike activity in the cluster IV neuron (Fig. 10, upper two traces). If burst activity in the cluster IV neuron results from excitatory actions of input C, this would be in keeping with our earlier observations, viz. that suppression of spike activity in VD1 and RPD2 results in decreased activity of input C. When spike activity in VD1 was resumed, burst activity in the cluster IV neuron gradually increased again. In the case of cluster IV neurons, however, the effect appeared to be reciprocal: Suppression of spike activity in the cluster IV neuron resulted in decreased spike frequency in VD1 (Fig. 10, lower two traces).

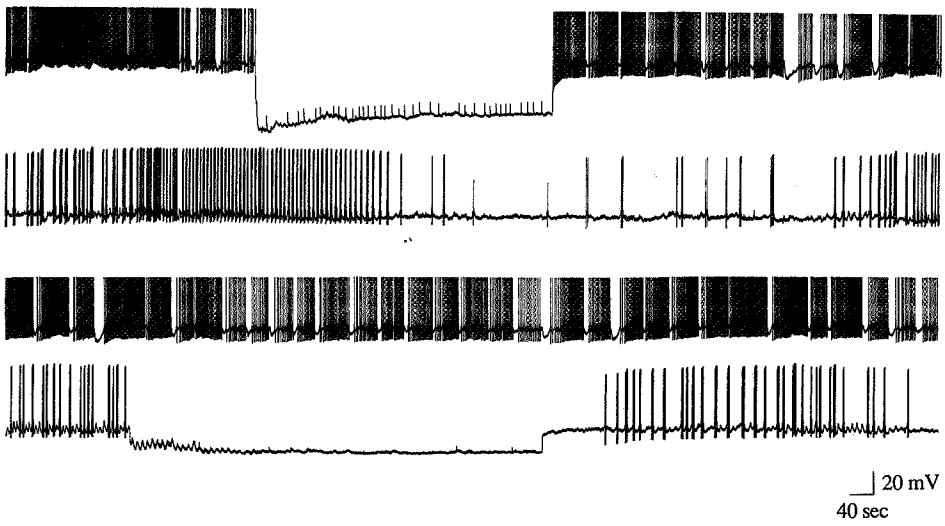


Fig. 10. Simultaneous recordings of a type IV neuron (upper trace) and VD1 (lower trace) in the isolated central nervous system. Suppression of spike activity in VD1 was, after a long latency, followed by a gradual hyperpolarization of the type IV neuron and decrease of burst activity until it was completely abolished. When VD1 was allowed to resume its normal spike activity, burst activity in the unidentified neuron resumed again. When the type IV neuron itself was hyperpolarized, the frequency of spike activity in VD1 slightly decreased. It increased again when the unidentified neuron was brought back to its resting potential and allowed to spike again.

The preceding observations are consistent with a model in which spike activity in VD1 and RPD2 and spike activity in cluster IV neurons are both necessary for the occurrence of input C. Patterned activity of input C would be the result of the joined activity of both types of neurons; cluster IV neurons could be characterized as contingent bursters, bursting activity being contingent upon activity of VD1 and RPD2. More data will be required, however, to substantiate this hypothesis, and to establish whether cluster IV neurons are actually implicated in the generation of input C activity.

Apart from the neurons, described above, two other neurons were found that are involved in control of pneumostome movements. They are not, however, involved in control of respiratory pneumostome movements. One (VD 4) is very likely to be involved in control of escape pneumostome movements, the other (VVPC) is very likely to be involved in control of contractions of the pneumostome which occur after mild mechanical stimulation of the pneumostome or mantle edge.

Control of escape pneumostome movements

VD 4 caused pneumostome movements upon suprathreshold electrical stimulation. We mentioned this neuron already in a previous paper (Janse *et al.*, 1985). The neuron is

located on the dorsal surface of the visceral ganglion, near the connective with the left parietal ganglion (Fig. 5). Its soma is much whiter than that of the surrounding neurons. It should be noted that a somewhat similar neuron is located in the same area of the visceral ganglion. In contrast to VD4, this neuron does not affect pneumostome movements. Morphologically and electrophysiologically, the two neurons are very similar. A difference that we have observed is that VD4 was always silent (n=12), while the other neuron could occasionally show very low, irregular spike activity. Due to the presence of these similar, but not identical neurons, identification of VD4 with certainty is not possible in the isolated CNS.

After a short depolarizing current (10 ms), VD4 responded characteristically with a burst of action potentials of about 5 s duration, which was abruptly terminated and followed by a clear post-burst hyperpolarization. The membrane potential gradually restored to its original, pre-pulse level. After a burst, a subsequent burst could not be elicited within a period of approximately 2 min. The burst of action potentials was always accompanied by a rapid, complete opening movement of the pneumostome, immediately followed by closure. Closure often started while the burst of VD4 was still in progress. In this respect the escape pneumostome movement, following burst activity of VD4 differed from the respiratory pneumostome movement, following spike activity in VDR neurons, where the delay between the opening movement and the subsequent closure was usually much longer. VD4 was sensitive to strong mechanical stimulation of the pneumostome and the mantle edge. A strong mechanical stimulation of the mantle edge elicited a similar burst of action potentials as evoked during electrical stimulation. It was also followed by a refractory state. This is shown in Fig. 11 A, where the response of RPD1 can be seen to be nearly identical during the three presentations of the mechanical stimulus, while the response of VD4 was completely absent during the second and third presentation of the stimulus.

The burst of acitivity in VD4 is followed by inhibition in several other types of neurons (Janse *et al.*, 1985). In addition to these connections, it was found that VDR neurones are inhibited with a delay of *ca.* 2 s after the onset of a burst of activity in VD4 (Fig. 11 B).

VD4 received inhibitory synaptic input during contractions of the pneumostome (input C) (Fig. 11 C); it did not receive synaptic input in common with the excitatory input on VDR neurons, accompanying respiratory movements of the pneumostome (input R). Lowering of the saline level and subsequent decrease of the PO_2 in the peripheral compartment did not affect the activity of VD4. A decrease of the PO_2 of the saline of the central compartment did not result in any change of the activity either.

LY fillings revealed branches of VD4 in the left parietal nerve (NPS), and the anal nerve (NA). Fine arborizations were primarily observed in the VG and LPG, close to the connective between both ganglia.

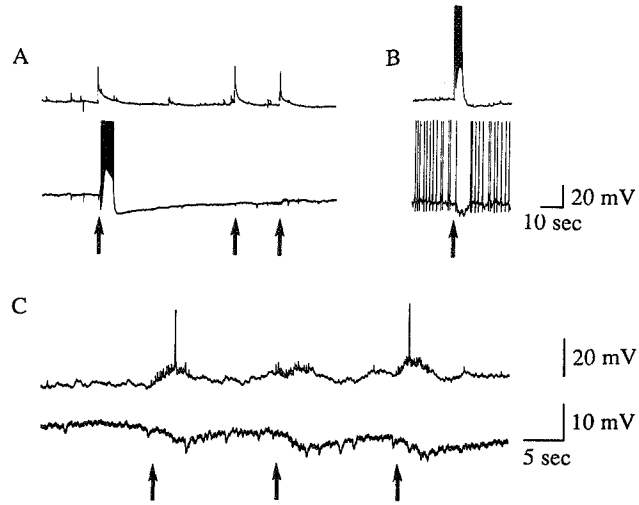


Fig.11. A. Simultaneous recording of the giant neuron RPD1 (uppertrace) and VD4 (lower trace). Arrows indicate the moment of strong mechanical stimulation of the mantle edge. The first stimulus elicited a compound epsp in RPD1 and a burst of action potentials in VD4, accompanied by opening and closure, respectively of the pneumostome. The second and third, identical stimuli still elicited the compound epsp in RPD1, but failed to elicit a burst in VD4 and the opening response of the pneumostome. B. Simultaneous recording of VD4 (upper trace) and a VDR neuron. A burst of action potentials in VD4 was after *ca.* 2 s followed by inhibition of the VDR neuron. C. Simultaneous recordings of a neuron from the A-group (hyperpolarized, upper trace) and VD4 (lower trace). Arrows indicate the occurrence of input C, resulting in single epsp's in the A-group neuron and a hyperpolarization in VD4. A, B and C are recordings made in semi-intact preparations.

These properties of VD4 are compatible with a function in escape behaviour: It is sensitive to stimuli that in the intact animal evoke an escape response, and causes rapid, complete opening followed by closure of the pneumostome, similar as to the pneumostome movement that is part of the escape response in *Lymnaea*.

Control of pneumostome contractions following mechanical stimulation of pneumostome or mantle edge

At the ventral surface of the VG, a neuron was found which upon suprathreshold depolarization caused contraction of the pneumostome, resulting in flattening of the outer flap against the mantle edge. This neuron will be referred to as Visceral Ventral Pneumostome Contraction neuron (VVPC). The VVPC neuron is certainly not the origin of input C: it did not show bursts of activity with a frequency similar to the occurrence of input C. Rather, it showed rather regular spike activity at low frequency (Fig. 12 A). Moreover, pneumostome contractions could occur in the absence of spike activity in the VVPC neuron (Figure 12 B, left panel; asterisk indicates occurrence of contraction of the pneumostome). This neuron did not receive synaptic input associated with regularly occurring contractions or respiratory movements of the pneumostome (inputs C and R).

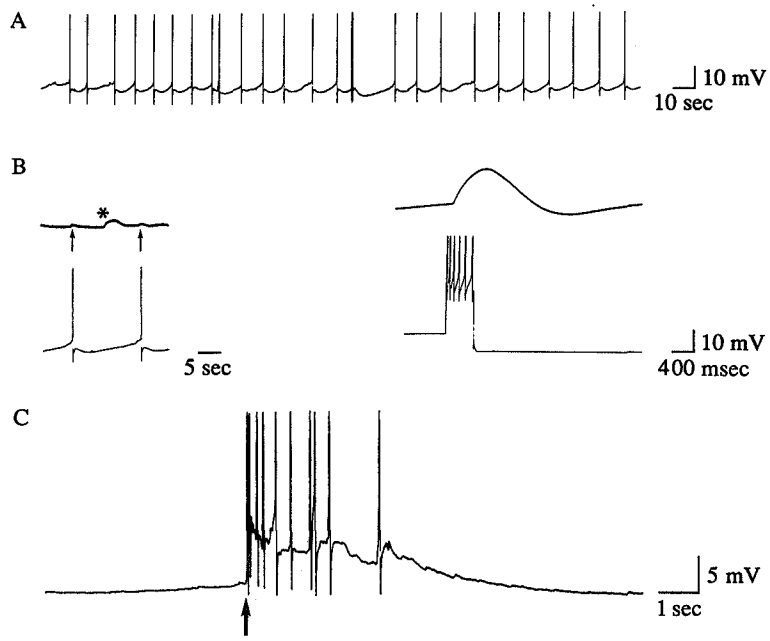


Fig. 12. Intracellular recording of the VVPC neuron. A. Representative recording of spike activity of the neuron in the semi-intact preparation. B. Simultaneous recordings of the VVPC neuron (lower trace) and pneumostome movements (upper trace). The left panel shows that single spikes in this neuron were accompanied by a small contractile movement of the pneumostome (arrows). Between the two spikes a stronger closure movement of the pneumostome occured (asterisk), which was not accompanied by the occurrence of synaptic input in this neuron. The right panel shows that a square wave, suprathreshold depolarizing stimulus, applied to VVPC resulted in the generation of a number of spikes and concomitant contraction of the pneumostome. C. Mechanical stimulation of the mantle edge (arrow) resulted in the occurrence of compound epsp's in VVPC, depolarization of the membrane potential and the generation of spikes. A, B and C are recordings from the same preparation.

Its activity was not affected by internal- or external changes in PO_2. The soma of this neuron is located in the centre of the ventral surface of the VG, approximately at the point where the extended lines from the connective with the LPG and RPG would intersect (Fig. 5).The soma is *ca.* 80 µm in diameter and pale orange in colour. Each spike was accompanied by a slight contractile movement of the pneumostome (Fig. 12 B, left panel). Fig. 12 B (right panel) shows a recording of the contracting movement of the pneumostome in response to depolarization of the VVPC neuron. The amplitude of the contraction depended on the frequency of firing of this neuron. The VVPC neuron was sensitive to mechanical stimulation of the pneumostome and mantle edge, which caused excitation of the neuron, accompanied by contraction of the pneumostome (Fig. 12 C). LY stainings of this neuron revealed projections into the NA.

Table 2. Summary of the post-synaptic effects of inputs C and R and effects of decreased PO_2 in the mantle- and lung area on central neurons, putatively involved in control of different types of pneumostome movements and on central neurons, showing spike activity correlated with pneumostome movements (- : no effect).

Cell type:	Putative role of neurons in control of pneumo-stome movements:	post-synaptic effects of input C:	input R:	effect of decreased PO_2:
VDR	control of respiratory movements	inh.	exc.	exc.
VD1, RPD2	control of respiratory movements	exc.	inh.	inh.
VD4	control of pneumostome movements associated with escape of the animal	inh.		
VVPC	control of pneumostome movements associated with with-drawal of the animal			
G - group	none; spike-activity merely correlated with pneumostome movements	inh.	exc.	exc.
A - group	none; spike-activity merely correlated with pneumostome movements	exc.		

The effects of central neurons on pneumostome movements and the effects of inputs C and R and of a decrease of the PO_2 in the lung-mantle area on these neurons are summarized in Table 2.

DISCUSSION

In the intact animal, movements of the pneumostome are part of three different types of behaviour. They occur as part of respiratory behaviour, as part of a general withdrawal reaction of the animal and as part of escape behaviour (chapter 2). Respiratory movements of the pneumostome usually consist of one or two opening movements, each followed by closure. Pneumostome movement during withdrawal consists of a contraction of the pneumostome; when, at the time of withdrawal the pneumostome was open, it can be observed as a complete closure movement. When the pneumostome was

already closed, a movement can still be observed, consisting of flattening of the outer edge of the pneumostome against the mantle. During escape, the pneumostome movement consists of a rapid complete opening, followed by rapid closure. The present data show that each of these functionally different types of pneumostome movements also occur in the semi-intact preparation. Central neurons could be subdivided into groups that show properties which are appropriate for a regulatory role in one of these behaviours.

Control of pneumostome movements that serve a respiratory function

Among the neurons that affect pneumostome movements, VDR neurons and VD1 and RPD2 are likely to be involved in control of respiratory movements. This follows from the observations that they are sensitive to changes of the saline level in the peripheral compartment as well as to changes in the PO_2 of the peripheral compartment. The changes in the saline level in the peripheral compartment probably mimick the stimuli which are experienced by the intact animal during surfacing and submergence. In the intact animal, these stimuli are capable of inducing opening and closure of the pneumostome, respectively. In the intact animal too, the number and duration of pneumostome opening movements strongly depend on PO_2 of the inspired air (chapter 2). Involvement of VD1 and RPD2 in control of respiratory movements is corroborated by recent findings of Kerkhoven *et al* (1991). They showed axonal projections of these neurones to the region of the pneumostome, osphradium and heart.

The activity of the VDR neurons and of VD1 and RPD2 appear to be determined to a large extent by the activity of input C and R, which have opposing effects on these cells. The VDR neurons probably play an important role in mediating the effects of the two inputs to the pneumostome. Increased spike frequency in these neurons is associated with increased amplitude of opening movement of the pneumostome, suggesting that these neurons are relatively directly involved in control of pneumostome movements. Based on the results of the electrical stimulation of whole nerves, one would expect motoneurons of the pneumostome to project into the NA and NPDE. LY stainings revealed that VDR neurons do not project into these nerves. Hence, it is most likely that these neurons have a pre-motor function, rather than a motor-function. After induction of opening of the pneumostome by electrical stimulation of a VDR neuron, closure of the pneumostome occurs after a variable latency. We do not know which neurons are involved in control of this closing movement. The closure movement is usually much more slowly as compared to the preceding opening movement, suggesting that some passive mechanisms may be involved as well.

Spike activity in VD1 and RPD2 is strongly correlated to movements of the pneumostome. This is caused by strong excitatory and inhibitory actions of input C and R, associated with contractions and respiratory movements of the pneumostome, respectively. In addition to this correlated activity, suppression of spike activity in VD1

and RPD2 results in increased frequency of respiratory movements of the pneumostome and decreased frequency of contractions of the pneumostome. By monitoring the activity of input C and R in follower cells, it appears that the effects that result from suppression of spike activity in VD1 and RPD2 can be explained by a decrease of the activity of input C and an increase of input R. This would imply that the connections between the interneurons, responsible for input C and R on the one hand and VD1 and RPD2 on the other, are reciprocal. There is a relatively long latency between the suppression of spike activity in VD1 and RPD2 and the change of activity of inputs C and R. The reason for this is as yet unknown, but it may mean that still other neurons are interposed. Within this network, a change of activity of VD1 and RPD2 can indirectly, through altered activity of inputs C and R, affect the activity of many neurons in the VG and RPG. The organization of the network is such, that the effects of the decrease of the peripheral PO_2 on pneumostome movements can be brought about by the decreased activity of VD1 and RPD2. It does not, however, rule out the possibility that the activity of the two inputs is also directly affected by input, related to changes in central or peripheral PO_2.

We observed that the pneumostome, as long as it is kept submerged, carries out regular contractions, resulting in flattening of the outer edge against the mantle. The function of these movements is unknown, but it is conceivable that they help to keep the pneumostome closed during submergence. These rhythmic contractions of the pneumostome are associated with activity of input C, whereas sustained activity of input C appears to require ongoing activity of VD1 and RPD2. Thus, the results so far suggest that VD1 and RPD2 are involved in generation of patterned neural activity, associated with contractions of the pneumostome. These contractions are not compatible with respiratory movements of the pneumostome. One possible way of preventing simultaneous activity of input C and input R is reflected in the actions of VD1 and RPD2 on both inputs: suppression of spike activity of VD1 and RPD2 decreases activity of input C, and increases activity of input R. Thus, VD1 and RPD2 may also be implicated in initiating respiratory movements of the pneumostome and simultaneously terminating the contractions of the pneumostome.

Input C appears to be very wide acting in the VG and RPG, and capable of producing different types of responses in follower cells. This is compatible with the findings of Benjamin and Winlow (1981) and Syed (1988), who described synaptic inputs which widely distribute throughout the CNS of *Lymnaea*. The effects of inputs C and R on follower cells do not fulfil all the criteria which should be met in order to identify the inputs with the ones, described by Benjamin and Winlow (1981) and by Syed (1988). The significance of the input on the relatively large number of neurons, that are probably not causally involved in control of pneumostome movements is not known.

The presence of two wide acting, antagonistic synaptic inputs, representing the major drives for respiratory activity, resembles the neural organization of respiratory pumping

in *Aplysia* (Byrne and Koester, 1978; Byrne, 1983; Koester and Koch, 1987; Alevizos *et al.*, 1991). Respiratory pumping is associated with activity of interneuron II (cells L25, 26 and 27), which is incompatible with activity of interneuron XI (cell L24). Based on our observations, similar functions can be tentatively attributed to the neurons, responsible for input R and C.

Control of pneumostome movements involved in escape behaviour

There are several reasons to suggest that VD4 is involved in control of pneumostome movements during escape behaviour. 1) Burst activity of the neuron is accompanied by a complete opening movement of the pneumostome, followed by closure, similar as to that observed in the intact animal during escape. 2) VD4 is sensitive to strong mechanical stimulation of all parts of the mantle area. 3) The response of VD4 to sensory stimulation is an all-or-none phenomenon. 4) The response of VD4 to stimulation habituates very quickly. In addition, we observed that a light-off stimulus to the semi-intact preparation is capable of eliciting a burst of action potentials in VD4. This also suggests a role of VD4 in mediating the escape response, since in the intact animal a shadow stimulus can elicit an escape response.

Some connections exist between VD4 and the neurons that are part of the respiratory network, described above. Input C produces slow inhibition in VD4, while burst activity in VD4 is followed by inhibition of VDR neurons. The latter coincides with the closure movement of the pneumostome and probably prevents opening of the pneumostome during this part of the escape response. Apart from these interactions, control of pneumostome movements that are part of respiratory- and escape behaviour is mediated by different neurons that act mostly in parallel.

Previously, bursts of activity in VD4 have been shown to be followed by inhibition or excitation in other neurons (Janse *et al.*, 1985). This arrangement of the network is consistent with the putative role of VD4 in escape, which involves simultaneous activation of several effector mechanisms and suppression of other types of activity.

Our data on VD4 are difficult to reconcile with those, presented by Syed (1988) and Syed *et al.* (1991a and b). According to these authors, burst activity of VD4 results in closure of the pneumostome, instead of initial opening, followed by closure. Comparing the data reveals, however, that the neuron, identified by Syed as VD4, can not be identical to the neuron which was first described by Janse *et al.* (1985) and was called VD4, which has properties that are consistent with those, presented here. Apart from the different effects of VD4 on pneumostome movements, there are two other differences: 1) morphology: the axonal projections of the neuron, described by Syed are confined to the ring of central ganglia, whereas the neuron, presented here projects in the left parietal and anal nerve. 2) electrophysiology: the neuron, described by Syed, shows spontaneous activity, whereas the neuron presented here, was always silent, and produced bursts of

action potentials only after electrical stimulation or appropriate stimulation of the preparation. The possibility, that two similar, but not identical neurons are located in the same part of the visceral ganglion, is corroborated by the fact that within one preparation, we have recorded from a neuron, which, by our standards would be VD4, and from a neuron which had similar properties, except that it did show spontaneous activity, and did not result in pneumostome opening upon burst activity. LY stainings of this neuron in several preparations revealed the same pattern of axonal projections, as described by Syed. If our hypothesis is correct, this would mean that our data and the data of Syed are complementary, instead of contradictory. As the properties of the neuron, described here, are consistent with those, presented by Janse *et al.* (1985), we maintain the identification of the present neuron as VD4.

Control of pneumostome movements involved in withdrawal

The properties of VVPC are consistent with a role in withdrawal. It produces, upon electrical stimulation, in a frequency dependent way, closure movements of the pneumostome. VVPC receives strong excitatory input upon mechanical stimulation of the pneumostome and mantle area. Input C and R do not affect the activity of this neuron. Thus, it appears that VVPC functions independently from the neural network, involved in control of respiratory movements of the pneumostome. We do not know whether a relationship between neurons that are tentatively implicated in control of withdrawal and escape, since we do not know whether connections exist between VVPC and VD4. Both neurons are sensitive to mechanosensory information, originating in the lung- and mantle area, but are involved in control of opposite movements of the pneumostome. It was also observed by De Vlieger *et al.* (1976), that mechanosensory stimulation of the mantle- and pneumostome area could elicit either closure- or opening movements of the pneumostome. In our experiments, mild tactile stimulation usually elicited closure movements, while stronger tactile stimulation resulted in an initial opening movement, followed by closure. If our hypothesis, that VVPC is involved in control of closure (withdrawal) and VD4 is involved in opening (escape), is correct, a possible explanation for the observed phenomena may be that the threshold for activation in VD4 is higher than in VVPC. We have not tested this hypothesis.

Peripheral components in control of pneumostome movements

In addition to the central components involved in control of pneumostome movements, there are also peripheral components. This follows from the observation that, in the absence of the CNS, the pneumostome is capable of producing completely coordinated closure- and opening movements. Cutting the connections with the CNS, only resulted in the loss of the response of the pneumostome to changes in PO_2 and the loss of the rhythmically occurring contractions. The loss of the rhythmic contractions is in keeping

with the observation that these movements result from the activity of input C, which originates in the VG. In the previous section, we suggested that the response of central neurons to a decreased peripheral PO_2 is mediated by increased activity of input R, which also originates within the VG. The loss of the responses of the pneumostome to changes in PO_2 supports this hypothesis, and suggests that this central pathway is an absolute requirement for these responses of the pneumostome.

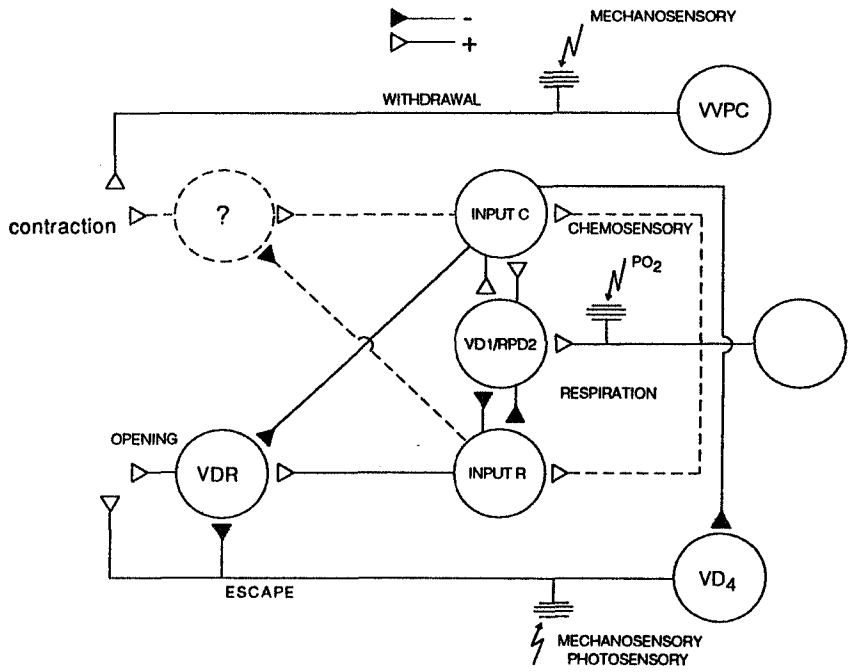


Fig. 13. Schematic representation of the possible organization of the central neural network, involved in control of pneumostome movements. For details see text. The neurons, responsible for input C and R, are tentatively considered as the major interneurons, involved in control of respiratory movements of the pneumostome. They have in the majority of cases opposing actions on follower cells in the VG and RPG. The latter are considered to be putative premotor neurons. Follower neurons that cause closure of the pneumostome and that are equivalent to VDR neurons are included in the network, although experimental data for the existence of such neurons remains to be established. They are included in the network, as the rhythmic closure movements of the pneumostome, associated with the occurrence of input C, are abolished when central synaptic transmission is blocked by high Mg^{2+}- low Ca^{2+} saline. Our data are consistent with reciprocal connections between neurons, responsible for input C and R on the one hand, and VD1 and RPD2 on the other hand. In this model, input that is related to changes in PO_2 is addressed to VD1 and RPD2. Possibly this input acts also directly on the interneurons C and R, which is also tentatively included in the model. The network appears to function largely independent from the neurons, involved in control of pneumostome movements that are associated with escape and withdrawal.

Our interpretation of the data gives rise to a model of the possible organization of the neuronal network, involved in control of each of the functionally different types of pneumostome movements; this model is presented in Figure 13.

From the preceding sections, it follows that the muscles, capable of producing opening- and closure of the pneumostome, participate in multiple behaviours. The involvement of a single effector organ in generation of multiple behaviours has been frequently documented. With respect to control mechanisms, single neural networks have been demonstrated to be capable of producing flexible motor outputs in a number of cases (Mpitsos and Cohan, 1986; Croll and Davis, 1987; Getting and Dekin, 1985). In the case of the different types of behaviour in which the pneumostome of *Lymnaea* participates, the situation is apparently different. The motor programme for the pneumostome movements in the different behaviours does not seem to originate from a single neural network, capable of producing a flexible outcome, but rather from largely separate neural pathways.

REFERENCES

Alevizos A, Weiss, K. R. and Koester J. (1991). Synaptic actions of identified peptidergic neuron R15 in *Aplysia*. I. Activation of respiratory pumping. J. Neurosci. **11**: 1263 - 1274.

Benjamin, P. R. and Winlow, W. (1981). The distribution of three wide - acting synaptic inputs to identified neurons in the isolated brain of *Lymnaea stagnalis* (L.). Comp. Biochem. Physiol. **70A**: 293 - 307.

Boer, H. H., Schot, L. P. C., Roubos. E. W., ter Maat, A., Lodder, J. C., Reichelt, D. and Swaab, D. F. (1979). ACTH - like immunoreactivity in two electrotonically coupled giant neurons in the pond snail (*Lymnaea stagnalis*). Cell Tiss. Res. **202**: 231 - 240.

Bogerd J. Generation of neuropeptide diversity by alternative splicing in two giant neurons, VD1 and RPD2, of the mollusc *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.

Byrne, J. and Koester, J. (1978). Respiratory pumping: neuronal control of a centrally commanded behavior in *Aplysia*. Brain Research **143**: 87 - 105.

Byrne, J. (1983). Identification and initial characterization of a cluster of command and pattern-generating neurons underlying respiratory pumping in *Aplysia californica*. J. Neurophysiol. **49**: 491 - 508.

Croll, R. P. and Davis, W. J. (1987). Neural mechanisms of motor program switching in *Pleurobranchaea*. In: S. P. Wise (Ed.), Higher brain functions: recent explorations of the brain's emergent properties. John Wiley, London.

Davis, W. J. (1985). Central feedback loops and some implications for motor control. In: W. J. P. Barnes and M. H. Gladden (Eds.), Feedback and motor control in invertebrates and vertebrates. Croom Helm, London.

Getting, P. A. and Dekin, M. S. (1985). *Tritonia* swimming: a model system for integration within rhythmic motor systems. In: A. I. Selverston (Ed.), Model neural networks and behavior. Plenum, New York.

Janse, C., van der Wilt, G. J., van der Plas, J. and van der Roest, M. (1985). Central and peripheral neurones involved in oxygen perception in the pulmonate snail *Lymnaea stagnalis* (Mollusca, Gastropoda). Comp. Biochem. Physiol. **82A**: 459 - 469.

Kerkhoven R. M., Croll R. P., van Minnen J., Bogerd J., Ramkema M. D., Lodder H and Boer H. H. (1991). Axonal mapping of the giant peptidergic neurons VD1 and RPD2 located in the central nervous system of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain research **556**: 8 - 16.

Koester, J. and Koch, U. T. (1987). Neural control of the circulatory system of *Aplysia*. Experientia **43**: 972 - 980.

Lever, A. J., Kraal, H. and de Vlieger, T. A. (1977). A behavioural and electrophysiological study of the withdrawal reaction of the pond snail *Lymnaea stagnalis* (L.) with particular reference to tentacle contraction. Proc. Kon. Ned. Akad. Wetensch. **C80**: 114 - 127.

Mpitsos, G. J. and Cohan, C. S. (1986). Convergence in a distributed nervous system: parallel processing and self-organization. J. Neurobiol. **17**: 517 - 545.

Slade, C. T., Mills, J. and Winlow, W. (1981). The organisation of neurones of the paired pedal ganglia of *Lymnaea stagnalis* (L.). Comp. Biochem. Physiol. **69A**: 789 - 803.

Syed, N. I. (1988). The neural control of locomotion in *Lymnaea*. Ph. D. Thesis, Department of Physiology, University of Leeds, U. K.

Syed N. I., D. Harrison and W. Winlow (1991a). Respiratory behaviour in the pond snail *Lymnaea stagnalis*. I. Behavioral analysis and the identification of motor neurons. J. Comp. Physiol. **169 A**: 541 - 555.

Syed, N. I. and W. Winlow (1991b). Respiratory behaviour in the pond snail *Lymnaea stagnalis*. II. Neural elements of the central pattern generator (CPG). J. Comp. Physiol. **169 A**: 557 - 568.

de Vlieger, T. A., Lever- De Vries, C. H. and Plesch, B. E. C. (1976). Peripheral and central control of the pneumostome in *Lymnaea stagnalis*. In: Neurobiology of Invertebrates. Gastropoda Brain. Tihany, 1975, pp. 629 - 634.

CHAPTER 8

EFFECTS OF HISTAMINE AND CATECHOLAMINES ON ELECTRICAL ACTIVITY OF PEPTIDERGIC NEURONS, INVOLVED IN CONTROL OF RESPIRATION IN THE FRESHWATER SNAIL *LYMNAEA STAGNALIS*

SUMMARY

1. By recording intracellulary we studied effects of putative neurotransmitters on the electrical activity of two electrotonically coupled giant neurons VD1 and RPD2. Experiments were done on the isolated central nervous system (CNS), semi-intact preparations and isolated neurons. In addition, effects of competitive receptor antagonists on endogenous synaptic activities were studied.

2. After isolation, RPD2 is usually silent, while VD1 shows regular spike activity. This suggests that the patterned activity of the two neurons as recorded in the CNS and in the semi-intact preparation is primarily determined by the occurrence of synaptic inputs.

3. In the previous chapter we described two inputs on VD1 and RPD2: Input C, which is excitatory and is associated with contractions of the pneumostome, and input R, which is inhibitory, and is associated with respiratory movements of the pneumostome.

3. The present data show that the effects of input C are probably mediated by histamine: application of histamine to VD1 and RPD2 and other follower cells mimicks this input; application of a histamine receptor antagonist blocks the occurrence of the excitatory input on VD1 and RPD2; application of histamine results in contraction of the pneumostome.

4. The actions of input R are probably mediated by catecholamines: application of dopamine (DA) or nor-adrenaline (NA) to follower cells of this input mimicks its actions; application of catecholaminergic receptor antagonists block the occurence of the endogenous input; application of DA or NA results in respiratory movements of the pneumostome.

INTRODUCTION

Lymnaea stagnalis shows an intermittent mode of breathing. Periods of aerial ventilation at the water surface are alternated by periods of apnoea during submergence. The number and duration of ventilatory movements during one surfacing period strongly depends on

the PO_2 of the inspired air. The duration of submergence between two successive ventilatory periods strongly depends on the PO_2 of the ambient water (chapter 2). Analysis of the neural network, involved in control of respiratory movements in *Lymnaea* has demonstrated that two peptidergic giant neurons, VD1 and RPD2 (nomenclature after Benjamin and Winlow, 1981) probably play an important role in control of respiratory movements (chapter 7). The two neurons are strongly electrotonically coupled, fire one - to - one action potentials and show ultrastructural features of neurosecretory neurons (Boer *et al.*, 1979). Both neurons branch extensively throughout the entire CNS. Axonal projections of these neurones have been demonstrated in the pneumostome-, osphradium- and heart region (Kerkhoven et al., 1991). The neurones produce cardioactive peptides that show homolgy with *Aplysia* R15 peptides (Bogerd, 1992). Their electrical properties have been extensively studied in relation to changes in age (Wildering, 1992).

The role of VD1 and RPD2 in control of respiratory movements was demonstrated in electrophysiological studies on semi-intact preparations(chapter 7). In this type of preparation, pneumostome movements occur which resemble the respiratory movements of the pneumostome in the intact animal. The occurrence of these pneumostome movements is associated with activity of a synaptic input (input R). The intervals between respiratory movements are associated with the activity of another synaptic input, antagonistic to input R (input C). Input C has an excitatory action on VD1 and RPD2, whereas input R has an inhibitory action. Exposure of the lung-mantle area of the semi-intact preparation to hypoxia results in decreased activity of input C and increased activity of input R, thus increasing respiratory activity. As a result of the change in activity of the two inputs, the activity of VD1 and RPD2 is strongly suppressed during periods of hypoxia. Inputs C and R probably constitute the major synaptic drives for respiratory activity. The effects of VD1 and RPD2 on the neuronal network, involved in control of respiratory movements could be explained through effects on the activity of inputs C and R.

In the present paper we describe the role of putative transmitters that mediate the responses of VD1 and RPD2 to the two synaptic inputs. Our results suggest that the actions of input C on VD1 and RPD2 are mediated by histamine. Input R on VD1 and RPD2 is probably mediated by catecholamines.

MATERIALS AND METHODS

Animals and preparations

Laboratory bred *Lymnaea stagnalis* (shell length *ca.* 30 mm) were used. Animals were kept at a 12h - 12 h light - dark regimen and an ambient temperature of 20 ^{o}C. They were fed lettuce every other day.

Prior to dissection, animals were anaesthetized by injection of 1 ml of 30 mM $MgCl_2$ in the foot. The central nervous system (CNS) was isolated and pinned down in a recording chamber (volume *ca.* 1 ml), lined with Xantropen (Bayer) and filled with saline. The connective tissue, covering the visceral- and right parietal ganglion was carefully removed with forceps to facilitate impalement of VD1 and RPD2. In some experiments, semi-intact preparations were used. These consisted of the mantle area, containing lung cavity, heart, kidney, pneumostome and adjacent areas of the skin connected to the CNS by the visceral and parietal nerves. The lung cavity was cut dorsally. This allowed exposure of the cavity to gas mixtures with different PO_2 (Janse *et al.*, 1985). Preparations were pinned down in a recording chamber, lined with Xantropen. The chamber consisted of two compartments, seperated from each other by a partition with the nerves running underneath. The partition was made air- and water tight with grease. Each compartment was filled with *ca.* 5 ml saline.

Procedures of isolation of central neurons

VD1 and RPD2 were isolated in a way, similar as described by Moed *et al.* (1989) for caudo dorsal cells. Each isolated neuron was transferred to a Petri dish (Costar), filled with HBS. The isolated neurons were allowed to attach to the bottom of the dish. Attachment usually occurred within 3 - 4 h after isolation.

Measurements

Intracellular recordings were made with glass micro electrodes (20 - 40 MΩ), filled with 0.5 M KCl. Signals were amplified, displayed and stored with conventional electrophysiological equipment.

In semi-intact preparations, pneumostome movements were measured with an optical device. PO_2 measurements were done with a polarographic oxygen electrode, connected to an amplifier (Instech). The electrode was calibrated with saline, saturated with pressurized air (20 % O_2) and with a solution of 0.01 M $Na_2S_2O_4$ (Merck, Darmstadt) (0 % O_2).

Application of transmitters and drugs

Transmitters and drugs were added to the bath containing the CNS or isolated neurons, in a concentration of 10 times the final concentration in the bath medium and mixed instantaneously. The concentrations mentioned in the text refer to final concentrations. A perfusion system consisting of a micropump and aspiration by means of vacuum allowed quick washing of the preparation with saline (circulation velocity *ca.* 4 ml.min-1).

In a number of experiments, transmitters and drugs were added ionophoretically. A WPI model 160 Microelectrophoresis Programmer was used, driven by a pulse generator made in our laboratory. During ionophoretic application the CNS was perfused with low

Ca^{2+}, high Mg^{2+} saline, to block synaptic transmission. Prior to ionophoretic application, the effects of the saline on synaptic transmission were determined in neurons, known to show synaptic connections (VD4 and neurons of the A-group). The electrodes were filled with solutions of 10^{-1} M of the transmitters or drugs.

Salines

Composition of the saline was as follows (in mM): NaCl, 30.0; KCl, 1.7; $NaCH_3SO_4$, 10.0; $MgCl_2.6H_2O$, 1.5; $CaCl_2.2H_2O$, 4.0; HEPES, 10.0; $NaHCO_3$, 5.0; adjusted to pH 7.8 with 3 N NaOH. Low Ca^{2+}-, high Mg^{2+} saline was of the following composition (in mM): NaCl, 30.0; KCl, 1.7; $NaCH_3SO_4$, 10.0; $MgCl_2$, 5.5; HEPES, 10.0; $NaHCO_3$, 5.0; adjusted to pH 7.8 with 3 N NaOH.

Chemicals used

Dopamine hydrochloride (DA), acetylcholine, gamma-amino butaric acid, 5 - hydroxytryptamine oxalate, DL-octopamine, L-glutamate sodium salt, all from Sigma, L-noradrenaline bitatrate (NA) from Serva and histamine dihydrochloride (HA), from Merck. Mepyramine, propranolol and (-)- sulpiride were gifts from Dr. R. P. M. Smits from the Department of Pharmacology, Vrije Universiteit, Amsterdam.

RESULTS

Activity patterns of VD1 and RPD2

In the isolated CNS and in the semi-intact preparation, VD1 and RPD2 always showed a fully synchronized activity. This implies that in these cases, activity of either RPD2 or VD1 could be monitored to describe responses of both cells.

In the isolated CNS the activity pattern of VD1 and RPD2 varied, depending on the activity of input C and input R. Usually, input C was predominantly active. In these cases, VD1 and RPD2 showed steady firing activity with regular periods of increased firing rate, due to input C. In preparations where input R was spontaneously active, spike activity was interrupted by periods of inhibition of varying duration (Fig. 1 A).

In the semi-intact preparation similar patterns of activity were observed. Activity of input C and R could, however, be affected by sensory stimulation of the preparation. Thus, exposure of the lung - mantle area to hypoxic air resulted in increased activity of input R and decreased activity of input C.

After isolation, VD1 showed regular firing activity with an average firing frequency of 1 Hz and a membrane potential of about -50 mV, whereas RPD2 was silent or showed irregular spiking activity at a membrane potential of approximately -70 mV (Fig. 1 B).

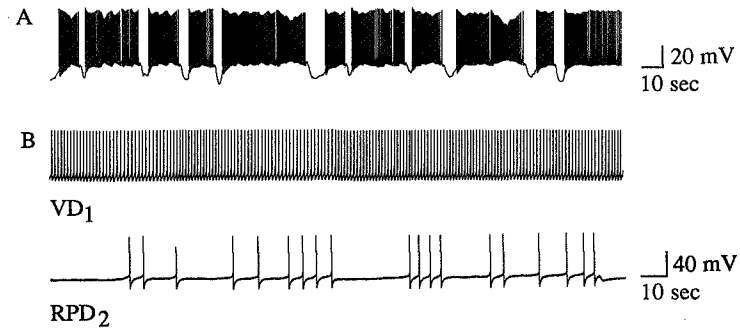


Fig. 1. Activity patterns of VD1 in the isolated CNS (A) and of VD1 and RPD2 after isolation (B). The hyperpolarizations in (A) were due to activity of input R.

These data show that on the one hand certain differences exist between the membrane properties of VD1 and RPD2, while on the other hand, both neurons show identical firing activity *in situ*.

Responses of VD1 and RPD2 to application of transmitters and drugs

In the following experiments effects of a number of pharmacologically active drugs on VD1 and RPD2 were studied. Putative transmitters (DOPA, ACh, GABA, octopamine, glutamate, NA and HA) and a number of their agonists and antagonists were used.

Putative transmitters with excitatory effects on VD1 and RPD2

From the substances tested, bath- or ionophoretic application of histamine (HA) invariably produced a powerful excitatory effect on VD1 and RPD2, both in isolated neurons (Fig. 2A) and in the isolated CNS. Threshold concentration of bath application of histamine was 10^{-7} M. The effect consisted of an increase of firing frequency and a concomitant depolarization. These effects occurred at concentrations of up to 10^{-6} M in a dose dependent way. At higher concentrations, the response could be subdivided into two parts: an initial, depolarizing response with short latency and of short duration, followed by a slow, long lasting depolarization of the membrane potential, accompanied by broadening of the action potential. The duration of the slow response varied from 2 to 3 min. The slow membrane depolarization and broadening of action potentials were more pronounced in RPD2 than in VD1 (Fig. 2B). This suggests that the properties of VD1 and RPD2 are not entirely identical.

Bath application of mepyramine (5.10^{-5} M), but not cimetidine (5.10^{-5} M) resulted in a decrease of the endogenous excitatory input (input C), accompanied by a decrease of the firing frequency of VD1 and a transient cessation of spike activity (Fig. 2 C). In the presence of the two histamine antagonists, application of histamine (10^{-6} M) did not

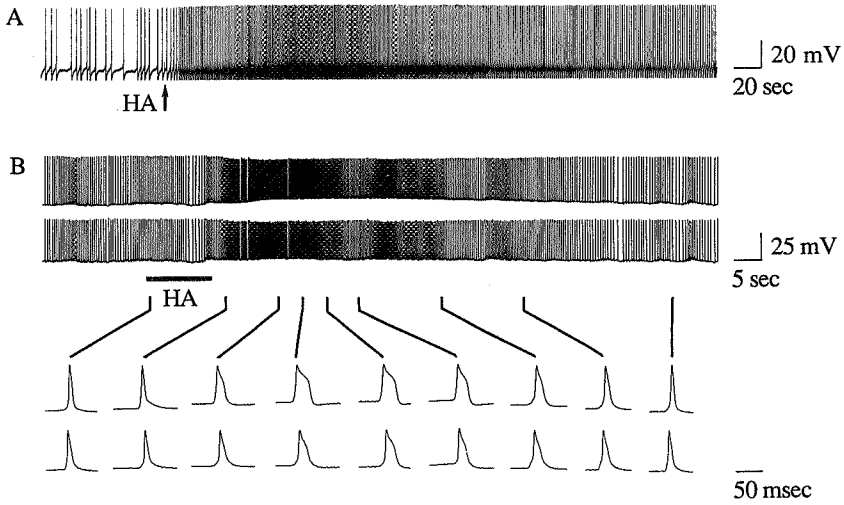


Fig. 2. Excitatory effects of HA in the isolated CNS and in isolated neurons. A. Effect of bath application of HA (10-7 M) on spike activity of isolated VD1. B. Effect of bath application of HA (10-5 M) on spike activity and shape of action potential of RPD2 (upper trace) and VD1 (lower trace), recorded in the isolated CNS.

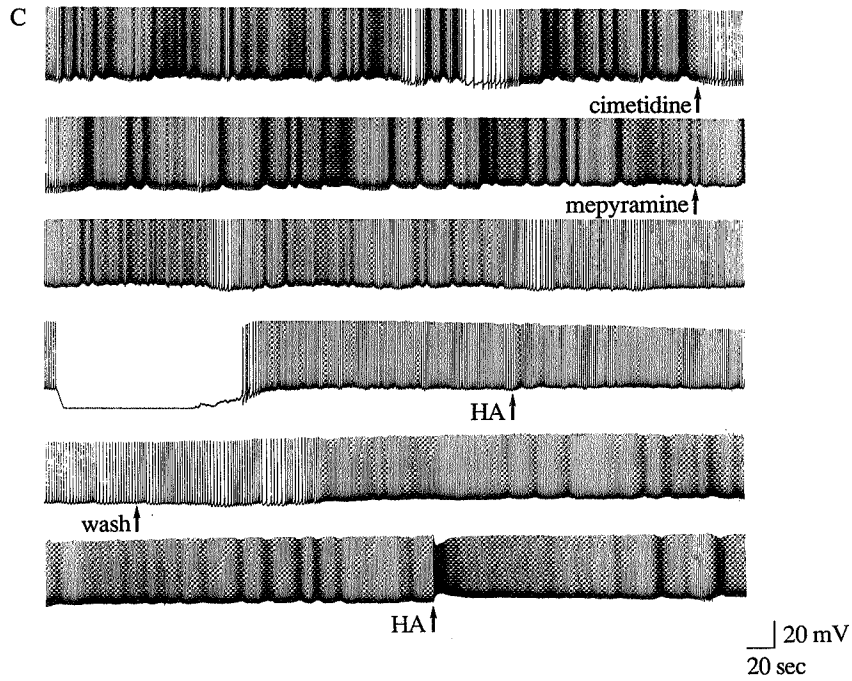


Fig. 2 C. Effect of bath application of cimetidine (5.10-5 M) and mepyramine (5.10-5 M) on endogenous excitatory synaptic input in VD1, recorded in the isolated CNS.

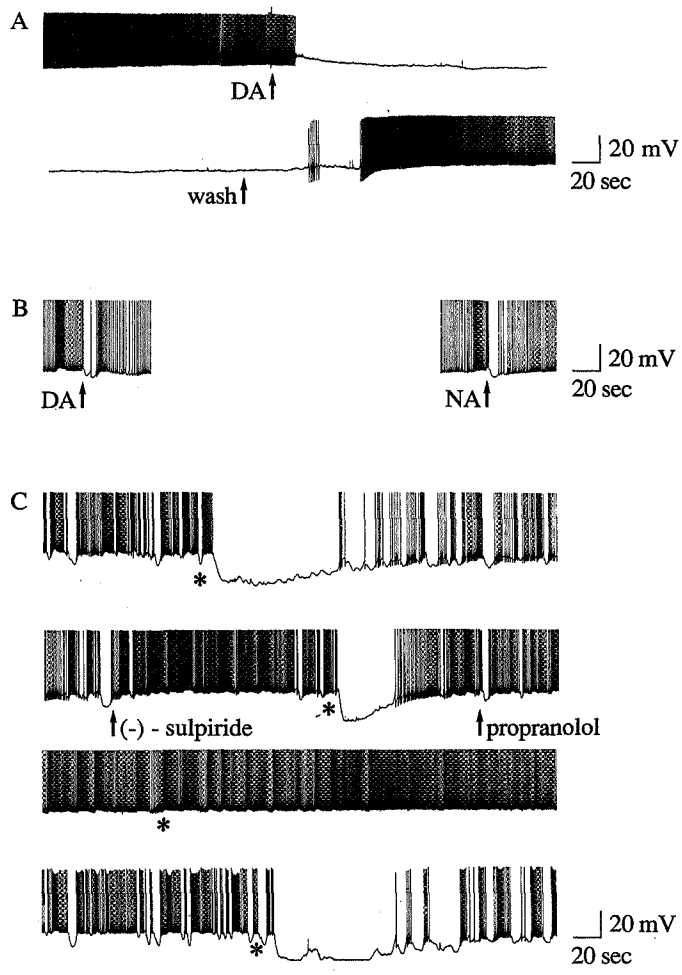


Fig.3. Effects of catecholamines on activity of VD1 and RPD2 in the isolated CNS and in isolated neurons. A. Effect of DA (10-7 M) on spike activity of an isolated RPD2. B. Effect of the application of DA (5.10-7 M), and effect of application of NA (5.10-7 M) on spike activity of RPD2 in the isolated central nervous system. C. Effect of induction of the endogenous inhibitory synaptic input in a semi-intact preparation by reduction of the peripheral PO_2 (marked by *) on spike activity in VD1: control, in the presence of (-)- sulpiride (10-5 M), in the presence of both, (-)- sulpiride and propranolol (both 10-5 M), and after wash.

produce the chatacteristic excitation of VD1. Washing the preparation with normal saline resulted in recovery of spike activity and reccurrence of the excitatory synaptic input after *ca.* 10 min. Also, the characteristic response of VD1 to histamine was restored.

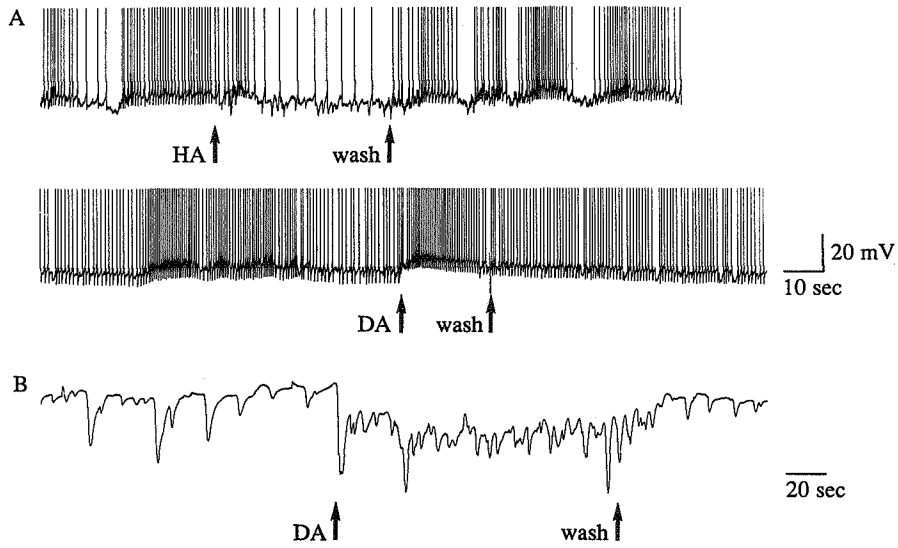


Fig. 4. A. Effect of bath application of HA and DA (both 5.10-7 M) to the CNS on spike activity of a VDR neuron. B. Effect of DA (10-5 M) application to the CNS on pneumostome movements in a reduced preparation.

Putative transmitters with inhibitory actions on VD1 and RPD2

From the substances tested, bath- and ionophoretic application of DA and NA caused a hyperpolarization and inhibition of spike activity of VD1 and RPD2 in isolated neurons (Fig. 3A) as well as in the isolated CNS (Fig. 3B). The threshold concentration of bath application was 10^{-7} M, which resulted in a hyperpolarization of about 5 mV of 5 - 10 s duration (*in situ*). Increasing the concentration of DA up to 10^{-4} M resulted in an increase of the amplitude and duration of the hyperpolarization.

The endogenous inhibitory synaptic input could not be blocked by application of either (--)sulpiride or propanolol (10^{-5} M) alone. The simultaneous application of both blockers (10^{-5} M), however, effectively blocked the endogenous inhibitory synaptic input. Recovery of the endogenous input occurred after *ca.* 5 min of wash (Fig. 3 C).

Effects of HA, DA, and NA on the activity of other follower cells of input Cand R and on pneumostome movements

The above findings indicate that the effects of inputs C and R are mediated by HA and DA and NA, respectively. In order to test this hypothesis, we assessed the effects of these compounds on other follower cells of these inputs and on pneumostome movements in the reduced preparation.

The effects of bath application of HA and DA (both 5.10-7 M) on the activity of a VDR neuron were studied. The VDR neurons are located in the rostral part of the dorsal surface of the visceral ganglion and is inhibited by input C and excited by input R (chapter 7). Fig. 4 A shows that HA had an inhibitory effect, whereas DA had an excitatory effect on this neuron.

A-group neurons, located in the RPG are excited by input C, whereas they do not receive input R (chapter 7). HA (5.10-7 M) had an excitatory effect on these neurons, whereas DA and NA (both 5.10-7 M) did no affect these neurons (not shown).

In the semi-intact preparation, application of DA or NA (10^{-5} M) to the compartment containing the CNS resulted in immediate opening of the pneumostome, followed by closure. As long as DA or NA were present, the frequency of opening movements of the pneumostome was increased (Fig.4B). Application of HA (10-5 M) resulted in closure of the pneumostome (not shown).

These data suggest that indeed, the actions of input C are mediated by HA, whereas the actions of input R are mediated by DA and NA.

DISCUSSION

Our results show that the bursting activity of VD1 and RPD2, observed in the isolated CNS and in semi-intact preparations, can be attributed to the activity of two synaptic inputs with opposing actions. It is probably not an endogenous property of the two neurons, as none of the two neurons appear capable of generating burst activity after isolation.

The actions of the excitatory synaptic input on VD1 and RPD2 (input C) are probably mediated by HA. This is based on the observation that application of HA in physiological concentrations mimicks the actions of the excitatory input on VD1 and RPD2. In addition, the endogenous excitatory synaptic input is blocked by application of the H1 receptor antagonist, mepyramine. The actions of the inhibitory input on VD1 and RPD2 (input R) are probably mediated by NA and DA. This is based on the observations that application of one of these transmitters in physiological concentrations mimicks the effects of the inhibitory input on VD1 and RPD2. In addition, the endogenous inhibitory synaptic input is blocked by application of a combination of the D2- and ß- receptor antagonists, sulpiride and propranolol.

The suggestion that the actions of input C are mediated by HA is further substantiated by the observation that HA mimicks the actions of input C on other follower cells, and causes closure of the pneumostome when applied to the CNS. HA has been shown to act as a neurotransmitter in several gastropod species, including *Lymnaea* (Turner and Cottrell, 1980). The actions of HA on follower cells in these species have been shown to be excitatory, inhibitory or biphasic (Ono and Mc Caman, 1985).

The observation that application of DA and NA to the CNS mimicks the actions of input R on other follower cells and causes opening of the pneumostome supports the suggestion that these transmitters mediate the actions of input R. Both DA and, to a lesser extent, NA have been shown to act as neurotransmitters in the CNS of several gastropod species (Rosza, 1984). In the semi-intact preparation, the activity of input R has been shown to increase during low PO_2 in the lung-mantle area (chapter 7). Interestingly, indications have been ontained that synaptic input, related to peripheral PO_2 on statocyst cells of Lymnaea is mediated by catecholamines as well (Janse *et al.*, 1988). The present data suggest that both transmitters appear to play an equally effective role in mediating the inhibitory synaptic input upon hypoxic stimuli.

Application of mepyramine often results in complete suppression of spike activity of VD1 and RPD2 and strong hyperpolarization. This can not be explained by a blockade of the effects of input C alone. It suggests that application of mepyramine results in enhanced activity of input R. These effects can be explained by the connectivity in the network, formed by VD1, RPD2 and inputs C and R (chapter 7): Previously, we have demonstrated that suppression of spike activity of VD1 and RPD2 resulted in an increase of the activity of input R and a decrease of input C. These findings were interpreted as an indication that the connections between VD1 and RPD2 on the one hand and the presynaptic neurons, responsible for input C and input R on the other hand are probably reciprocal. This might explain that suppression of the activity of VD1 and RPD2 results in additional inhibition of VD1 and RPD2 as a result of the increase of the activity of input R.

REFERENCES

Benjamin, P. R. and Winlow, W. (1981). The distribution of three wide - acting synaptic inputs to identified neurons in the isolated brain of *Lymnaea stagnalis* (L.). Comp. Biochem. Physiol. **70A**: 293 - 307.

Boer, H. H., Schot, L. P. C., Roubos. E. W., ter Maat, A., Lodder, J. C., Reichelt, D. and Swaab, D. F. (1979). ACTH - like immunoreactivity in two electrotonically coupled giant neurons in the pond snail (*Lymnaea stagnalis*). Cell Tiss. Res. **202**: 231 - 240.

Bogerd, J. Generation of neuropeptide diversity by alternative splicing in two giant neurons, VD1 and RPD2, of the mollusc *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.

Janse, C., van der Wilt, G. J., van der Roest, M. and Pieneman, A.W. (1988). Intracellularly recorded responses to tilt and efferent input of statocyst sensory cells in the pulmonate snail *Lymnaea stagnalis*. Comp. Biochem. Physiol. **90A**: 269 - 278.

Kerkhoven, R. M., RP Croll, J v Minnen, J Bogerd, MD Ramkema, H Lodder en HH Boer (1991). Axonal mapping of the giant peptidergic neurons VD1 and RPD2 located in the central nervous system of the pond snail *Lymnaea stagnalis*, with particular reference to the innervation of the auricle of the heart. Brain research **556**: 8 - 16.

Moed, P. J., Bos, N. P. A. and ter Maat, A. (1989). Morphology and electrophysiological characteristics of caudodorsal cells of *Lymnaea stagnalis* in dissociated cell culture. Comp. Biochem. Physiol. **92 A** (3): 445 - 453.

Ono, J. K. and Mc Caman, R. E. (1985). The central nervous system of *Aplysia californica:* A model system for cellular studies of central neurotransmission. In: A. I. Selverston (Ed.), Model Neural Networks and Behavior. Plenum, New York.

Rózsa, K. S.- (1984). The pharmacology of molluscan neurons. Progress in Neurobiology **23**: 79 - 150.

Turner, J. D. and Cottrell, G. A. (1977). Properties of an identified histamine - containing neurone. Nature **267**: 447 - 448.

Wildering W. C. Synaptic plasticity in the central nervous system of adult *Lymnaea stagnalis*. Thesis, Vrije Universiteit, 1992.